Biogeography of *Nothofagus* subgenus *Fuscospora* in the South Island of New Zealand inferred from chloroplast DNA

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Abstract

Nothofagus ('southern beech') is a major component in forests throughout the South Island of New Zealand. However all four species are disjunct across the central portion of the island. Hypotheses for the disjunction include the following explanations based on vicariance: (i) 'glacial refugia' hypothesis, where distributions have not recovered following elimination during the Pleistocene glacial period; (ii) 'environmental barriers' hypothesis, where edaphic, climatic and migration barriers hinder *Nothofagus* migration; and (iii) 'lateral plate shift' hypothesis, where populations have been rafted apart by lateral movement on the Australia-Pacific tectonic plate boundary since the early Miocene. Dispersal explanations imply non-survival south of the disjunction during glacial periods followed by seed dispersal during the Holocene. One dispersal hypothesis explains the presence of *N. fusca* and *N. truncata* in the south through long-distance pollen dispersal and hybridisation resulting in species 'reconstitution'.

A phylogeographic approach using sequences and RFLPs of non-coding chloroplast DNA (cpDNA) regions in the three New Zealand *Fuscospora* species is used to infer historic patterns of distribution and gene flow in the South Island. Maternally-inherited cpDNA is transmitted via seed, and is therefore appropriate for revealing variation at a geographically localised scale. Comparison of *N. fusca/N. truncata* and *N. solandri* cpDNA haplotypes south of the disjunction is used to assess the validity of the long-distance pollen dispersal and hybridisation hypothesis.

Evolutionary rates of cpDNA in *Fuscospora* was found to be very slow, in line with other studies. A single insertion in the *trn*L-*trn*F intergenic spacer was restricted to south of the disjunction. A very approximate estimate of its age was made, and it is unlikely to be more than 1-2 mya. It is unlikely to have occurred during the Holocene, indicating that *Fuscospora* populations have existed in isolation south of the disjunction throughout the last Pleistocene glacial period at least, and possibly much longer. Therefore a vicariance origin to the disjunction is favoured. Although no resolution can be made between the 'glacial refugia' and 'environmental barriers' hypotheses, the 'lateral plate shift' hypothesis is not supported. The southern South Island mutation is shared between all three species, suggesting that introgression of

the chloroplast genome has occurred, possibly in the absence of nuclear introgression. Assuming that *N. solandri* var. *cliffortioides* survived in the south during the last glacial period, this finding is compatible with scenarios of (i) *N. fusca*/*N. truncata* survival in the south; (ii) Holocene dispersal of *N. fusca*/*N. truncata* seed, and (iii) 'reconstitution' of *N. fusca*/*N truncata* via long-distance pollen dispersal and hybridisation.

1 General introduction

The genus *Nothofagus* ('southern beech') is of outstanding importance in the New Zealand biota. The four species, all endemic, are: *Nothofagus fusca* (red beech), *N. solandri* (mountain and black beech), *N. truncata* (hard beech) and *N. menziesii* (silver beech). Together they form the major component in New Zealand's indigenous forests. Of the total standing forest today, 68% consists of *Nothofagus*, either as pure forest (46%) or in mixtures with other tree species (22%) (Wardle 1984). *Nothofagus* is particularly significant in the South Island where, in pure and mixed forest, it makes up 84% of indigenous forest area, compared with 40% in the North Island (Wardle 1984). The genus is especially dominant in mountainous areas, and *N. solandri* var. *cliffortioides* and *N. menziesii*, in particular, form almost continuous subalpine forests in the axial mountains of both islands (Ogden et al. 1996).

Despite the overall dominance of *Nothofagus*, botanists have long been puzzled by its absence from several large areas which appear to have climatic and edaphic conditions suited to it. These distributional gaps include Stewart Island, the central part of the South Island, an area on the Tararua and Ruahine Ranges on either side of the Manawatu Gorge, and Mt Taranaki (Mt Egmont) (Wardle 1984; Ogden et al. 1996; McGlone et al. 1996). The existence of these gaps have posed questions for those trying to gain an understanding of the ecology of *Nothofagus*, and there has often been a tension between explanations based on ecological and historical causes.

1.1 The South Island 'beech gap'

The gap in the central South Island is more pronounced to the west of the Main Divide, where it is extends for 160 kilometres, and is commonly known as the Westland 'beech gap', To the east it is less pronounced, although a clear, shorter (around 75 kilometres) gap does exist, particularly in the proximity of the Southern Alps. The gap is common to all four *Nothofagus* species (Fig. 1.1). Although they are all abundant to the north of the gap, they vary in their abundance on the southern side, with one species (*N. truncata*) being limited to several isolated populations.

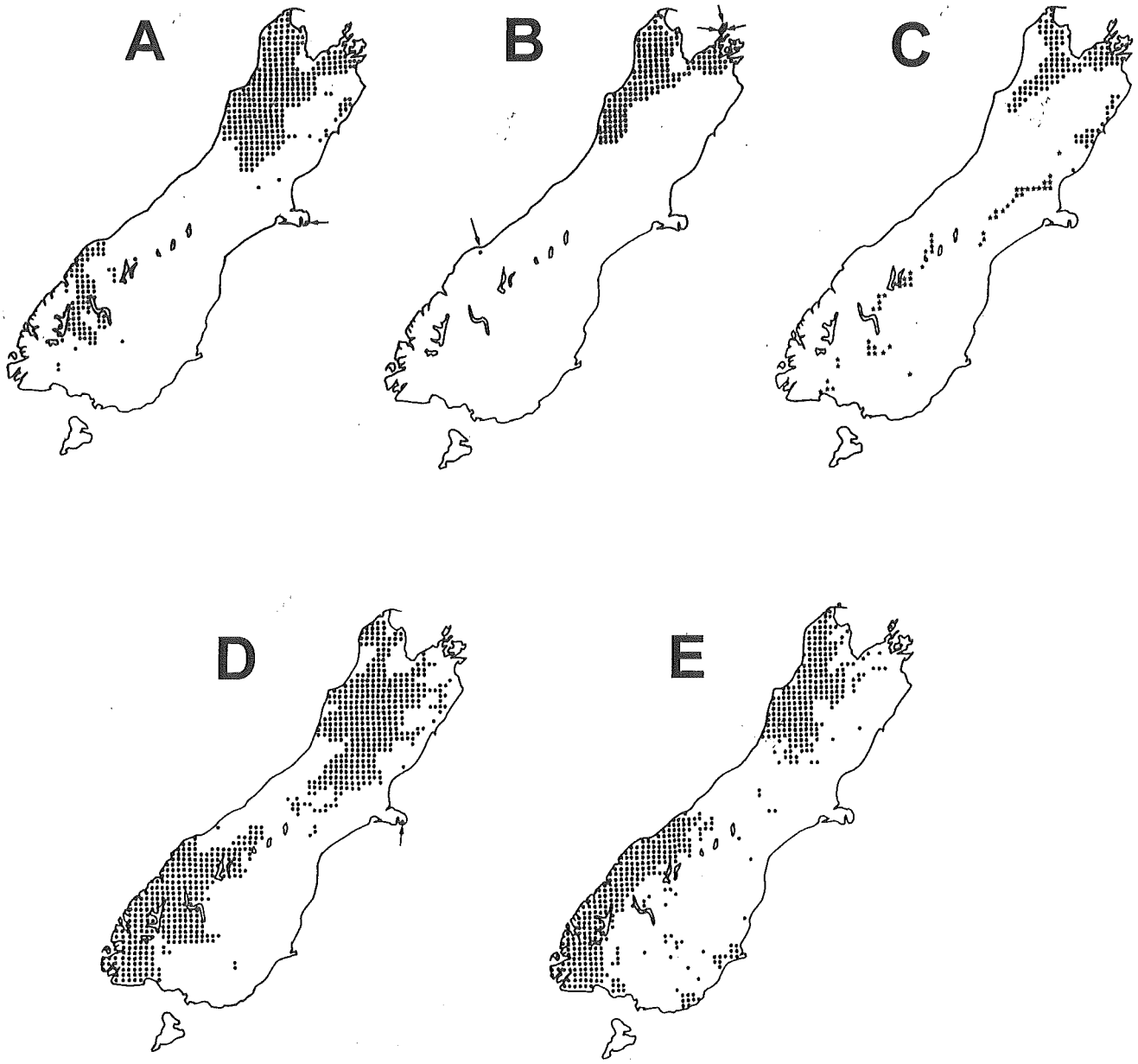

Fig. 1.1 Distribution of *Nothofagus* species in the South Island. (A) *Nothofagus fusca*; (B) *N. truncata*; (C) *N. solandri* var. *solandri*; (D) *N. solandri* var. *cliffortioides*; (E) *N. menziesii*.

Reproduced from Wardle (1984).

The gap in the central South Island can be labelled a 'disjunction', according to generally accepted definitions of the term. The definition of Cain (1944), that a disjunction is a space between populations wider than 'the normal dispersal capacity of the type', has been used in this debate (McGlone 1985). The 'normal dispersal capacity' of a species is open to interpretation, and in any case dispersal capabilities of most taxa are not sufficiently known to be able to allow a ready definition of 'normal dispersal distance' (Kloot 1984). This is true of *Nothofagus*, for which seed is usually wind dispersed. The seed has rudimentary wings which permit dispersal 'seldom more than a few hundred metres from the parent tree' (Wardle 1984). However there is strong evidence of occasional transport across at least 2.5 kilometres where strong wind events and topography combine to create updrafts (Burrows 1993). Uncommon but very severe storms could probably transport *Nothofagus* seeds across distances far greater than this. Even so, it seems clear that the size of the central South Island gap far exceeds the dispersal distance of *Nothofagus* seeds under normal conditions.

The central South Island disjunction is of of major biogeographic importance because of the physiognomic dominance of *Nothofagus* in forests in the north and south of the island (McGlone 1985). Its absence from the central regions demands an explanation, as it appears to be anomalous and does not readily conform to climatic and soil patterns (McGlone et al. 1996). Almost all hypotheses put forward to explain the disjunction are based on an origin by vicariance. Such scenarios envisage a more or less continuous distribution of *Nothofagus* across the South Island, until broken by an event, or series of events, in the central region of the island. Opinions differ on whether historical or ecological factors are primarily responsible. Arguments based on historical factors (notably pleistocene glacial periods) assume that current distributions are not stable, and that a gradual bridging of the gap is now occuring (Wardle 1963; Burrows 1965; Wardle 1988). On the other hand, hypotheses based on ecological factors suggest that edaphic and climatic conditions (possibly associated with long-term tectonic uplift) are the primary cause of exclusion, and that the boundaries of the gap are older and relatively stable (McGlone 1985; McGlone et al. 1996). Set against the popular vicariance theories, hypotheses of dispersal across the gap are not fashionable, although they cannot be totally discarded. This is particularly so given that *Nothofagus* distributions among southern continents, formerly explained

by vicariance events, are, in the light of recent palynological and molecular evidence, most parsimoniously explained by dispersalist theories (Hill and Dettman 1996).

2 *Nothofagus* disjunction across the central South Island

2.1 *Nothofagus* subgenus *Fuscospora*

Nothofagus is a southern hemisphere genus of forest trees and shrubs. Representatives are found in Australia, New Zealand, South America, New Guinea and New Caledonia (Wardle 1984; Hill 1996). The genus has traditionally been placed in the predominantly northern hemisphere family Fagaceae (Allan 1961). However recent work based on leaf morphology, and supported by inflorescence, pollen, chromosome and phytochemical data, suggests placement in a monogeneric family, Nothofagaceae (Jones 1986). Hill and Dettman (1996) conclude that the familial position will remain unresolved until more work is carried out on the ontogenetic development of *Nothofagus* inflorescences.

Four subgenera of *Nothofagus* (*Nothofagus*, *Fuscospora*, *Lophozonia* and *Brassospora*) are currently accepted (Hill and Dettmann 1996), although the relationships among them are still poorly understood (Hill 1996). The four subgenera were proposed by Hill and Read (1991), on the basis of differentiation in cupule morphology and leaf cuticular pattern. This infrageneric classification has since been supported by morphological (Hill and Jordan 1993), palynological (Hill and Read 1991) and both nuclear (Manos 1997) and chloroplast (Martin and Dowd 1993; Setoguchi et al. 1997) molecular data.

Three of the four New Zealand *Nothofagus* species, *N. fusca*, *N. solandri* and *N. truncata*, are representatives of the *Fuscospora* subgenus. The only other two members of *Fuscospora* are *N. alessandri* (Chile) and *N. gunnii* (Tasmania) (Hill and Dettmann 1996). The three New Zealand representatives are evergreen tree species, and form a complex with a relatively recent evolutionary divergence and a high rate of natural interspecific hybridisation (Wardle 1984). Ogden (1989) has referred to them as a coenospecies. The close relationship between the three is supported by a high degree of morphological similarity, for example in the leaf architecture (Hill and

Read 1991) and in their common revolute vernation, unique among *Nothofagus* (Philipson and Philipson 1979, 1988). They appear as an unresolved trichtomy in Hill and Jordan's (1993) cladogram based on a broad range of morphological characters. The close relationship is further reinforced by consistent similarities between the three in DNA sequences (Martin and Dowd 1993; Setoguchi et al. 1997; Manos 1997). Within *N. solandri*, a cline of variation runs from the small, elliptical-leaved *N. solandri* var. *cliffortioides* (mountain beech) normally found at high altitudes, to the larger, ovate-leaved *N. solandri* var. *solandri* (black beech) of lower altitudes (Wardle 1984; Ogden et al. 1996).

The only other *Nothofagus* species in New Zealand, *N. menziesii*, is a member of the subgenus *Lophozonia*. There are no records of hybridisation between it and the *Fuscospora* species (Wardle 1984; Ogden et al. 1996).

Fuscospora pollen first appears in the New Zealand record in the late Cretaceous or early Paleocene (McGlone et al. 1996). The importance of *Fuscospora* in the pollen record fluctated during the Cenozoic. From the late Miocene, *Fuscospora* increased in dominance, mirroring a corresponding decline in *Brassospora* (probably in response to climatic fluctuations) which finally disappeared from the record in the early Pleistocene. However the diversity of *Fuscospora* appears to have been low throughout (McGlone et al. 1996).

2.2 Discovery of the *Nothofagus* disjunction in the South Island

The existence of a disjunction in beech distributions in the South Island does not appear to have been apparent to early botanists. For example, Kirk (1889) described the abundance of the various *Nothofagus* species throughout the island, but made no mention of the absence of any of the beech species across the central part of the island. The gap was first noted by foresters (eg. Roberts 1909) carrying out comprehensive studies of distributions for timber values.

Over succeeding decades, the distribution of the beeches, and thus the extent of their disjunction, became refined as further vegetation studies of the Westland forests were

completed. For example, the distribution of *N. solandri* var. *cliffortioides* on the immediate southern side of the disjunction only gradually came to light as stands were found in isolated and scattered locations, as far north as Bald Hill (Holloway 1954). Most spectacularly, *N. truncata*, formerly believed to be confined to the north of the South Island, was discovered in 1977 in several small stands near Jackson Bay (June 1977), thus confirming a major South Island disjunction for all four of New Zealand's *Nothofagus* species.

2.3 Description of the *Nothofagus* disjunction in the South Island

For place names used in this section, refer to Fig. 2.1 (page 11).

For a more detailed description of these distributions, refer to Appendix 1 (page 117).

2.3.1 West of the Southern Alps

The disjunction on the western side of the Southern Alps applies to all four *Nothofagus* species. At neither edge of the disjunction is there an abrupt transition from beech-dominated forest to podocarp / broadleaved forest. On the north side, beech is dominant in Buller and the northern part of the West Coast, grading to forests dominated by podocarp / broadleaved hardwood species in the Grey catchment (Wardle 1984). Holloway (1954) observed that the forests north of the Grey and its tributary, the Ahaura, were largely beech with a smaller podocarp element, whereas to the south they were largely podocarp with a smaller beech component. About 12 km south of the Ahaura confluence with the Grey, beech becomes very restricted. The southern limit for all four species is the Taramakau valley (Wardle 1984).

At the southern edge of the disjunction there is a similar gradient in abundance, with beech first occuring very patchily. *N. menziesii* is first to appear, at the headwaters of the Karangarua River, about 160 km from the Taramakau River (Wardle 1984). South of the Paringa river, *N. menziesii* becomes dominant in mountain valleys inland (Holloway 1954). *N. solandri* var. *cliffortioides* first occurs in pockets just north of

the Waita River, and is only sporadic southwards from there (Holloway 1954). *N. fusca* first appears in the Arawata catchment where it is abundant (Holloway 1954), while *N. truncata* is confined to several small patches on low hills on the coastal plain between the Waiototo and Arawata rivers (June 1977). North of the Arawata, podocarp forests dominate on the low and often poorly-drained coastal strip, and beech occurs only patchily (Wardle 1980). South of the Arawata, beech or beech-podocarp forests dominate throughout. However distributions are complicated by geology, which includes large areas of swampy land and infertile substrates (Wardle 1984).

2.3.2 East of the Southern Alps

The picture of pre-human forest distribution east of the main divide has been obscured by burning, although we know that the disjunction is less well defined. The climate is much drier than on the West Coast, and this may account for the absence of *N. truncata* and the restricted distributions of *N. fusca* and *N. menziesii*. There are no verified records of *N. fusca* between the Waimakariri and the Hunter valleys, while *N. menziesii* is fairly widespread but never common. However *N. solandri* var. *cliffortioides* and forms intermediate with *N. solandri* var. *solandri* are widespread and dominant over large areas.

The disjunction is more marked close to the Southern Alps, where it extends for about 75 km. In the Rangitata catchment at the northern side of the gap *N. solandri* var. *cliffortioides* is the only beech species found. On the southern side of the gap, on the west side of the Tasman River and some distance southwards, the only beech species are *N. solandri* var. *cliffortioides* and *N. menziesii*. As on the West Coast the transition between beech-dominated forests and beech absence tends to be gradual, particularly on the north side of the disjunction.

Further east of the Southern Alps, the pattern is confused by the patchy distribution of *N. menziesii*, which is the only beech species found. The existence of sporadic populations suggests several small gaps rather than any single north-south disjunction.

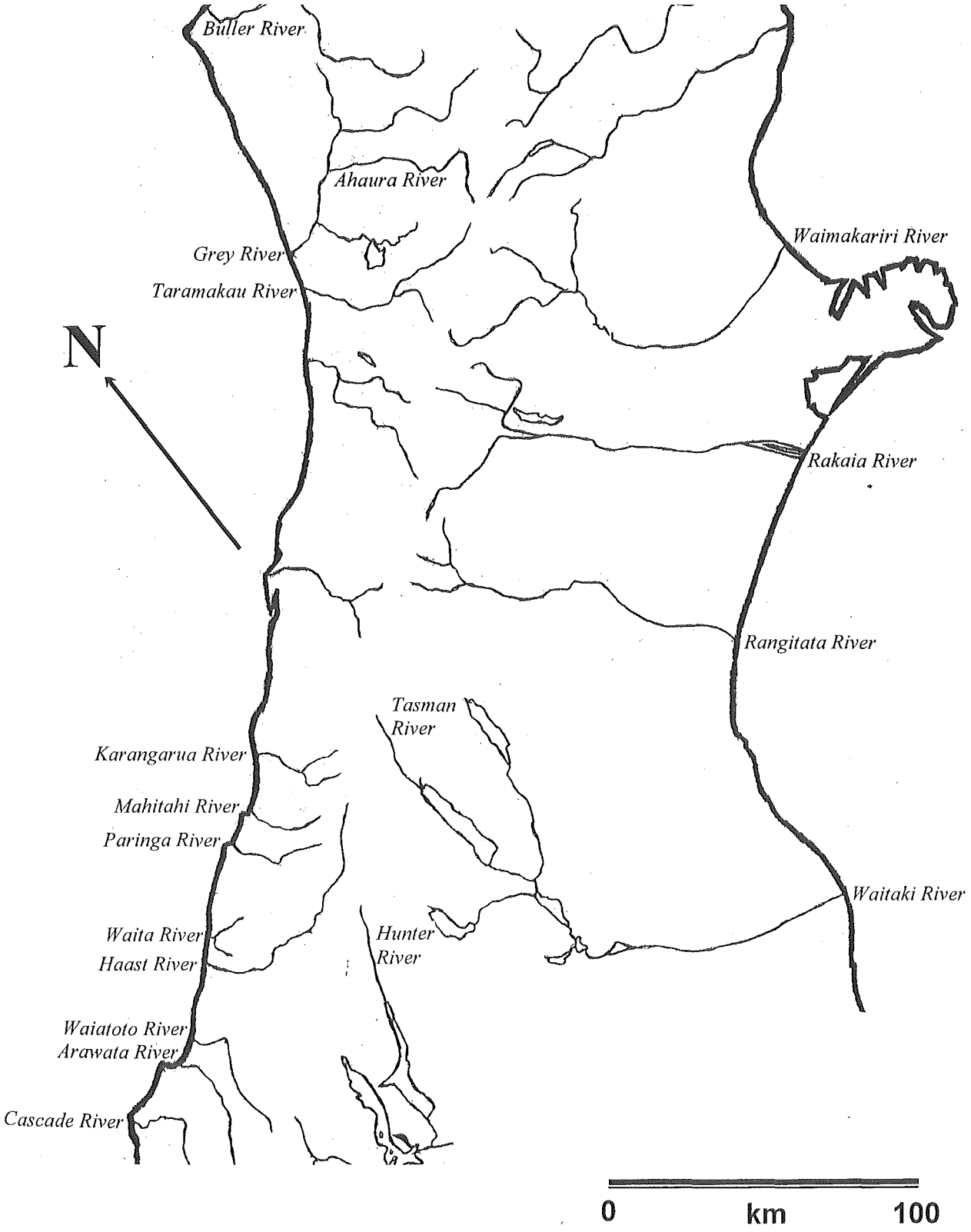

Fig. 2.1 Central South Island showing place names referred to in Section 2.3 and Appendix 1.

3 Hypotheses for the *Nothofagus* disjunction

Prior to the early 1960's, consideration of disjunctions in the South Island tended to concentrate on *Nothofagus* in particular. However, Wardle (1963) and Burrows (1965), recognised the existence of:

1. Recurring patterns of disjunction in the South Island, where a number of taxa (of which *Nothofagus* was only one) share similar ranges in the north and south of the island, and a similar gap in the central region, and

2. High rates of regional endemism in the northern and southern regions of the South Island, with an endemic-poor zone in the central part of the island.

Several general hypothesis have arisen on the basis that these two patterns have the same cause. Given that there is such a pronounced pattern of disjunctions and endemism over a number of taxa, it is very likely that there is a common cause for the majority, although the common cause may not necessarily apply to *Nothofagus*. Each species has its own unique ecology and dispersal capabilities, against which its disjunction should be evaluated. Accordingly, while the disjunction of *Nothofagus* should be evaluated against generalised theories, the picture for this taxa can only be elucidated from its ecology, historical record, molecular and other data. The generalised hypotheses do, however, provide a basis for assessing the disjunctions in *Nothofagus*, since it seems likely that one or more of them applies to *Nothofagus*, even if it doesn't apply to the majority of other taxa.

It is clear from an examination of the hypotheses, whether applied generally, or to *Nothofagus* in particular, that geological and climatic events have played a primary role in shaping modern-day distributions of plant (and animal) taxa in the South Island. The two main influences giving rise to the modern-day landscapes, and which are central to the debate over species distributions are:

- The imprints left on much of the South Island landscape by the repeated glaciation episodes of the Pleistocene.

- Plate tectonic activity, causing lateral displacement along the Alpine Fault and uplift of the Southern Alps.

3.1 Hypotheses for the disjunction due to vicariance

The most commonly advanced hypotheses are of a vicariance origin to the disjunction. These imply a formerly continuous distribution along the length of the South Island, and a subsequent break in the distribution in the centre of the island due to geological and/or climatic events.

3.1.1 'Glacial refugia' hypothesis

The 'glacial refugia' hypothesis predicts that taxa, formerly widespread or continuously distributed across the South Island, were eliminated from the central region due to climatic factors associated with the late Tertiary and Quaternary. (Wardle 1963; Burrows 1965). The major feature of this period was the repetition of approximately 20 cycles of alternate global warming (interglacial periods) and cooling (glacial periods) (Fig. 3.1) (Beu and Edwards 1984; Whitehouse and Pearce 1992). The glacial periods brought about the formation of extensive ice sheets over the central part of the South Island in particular (Fig. 3.2), and were accompanied by climates too severe for forest in most parts of the island (Section 3.3).

In the second half of the Pleistocene, since around 0.9 mya, the amplitude of cold/warm fluctuations increased, resulting in greater extents of ice sheets during glacial periods, and cooling-warming cycles developed a 100,000 year periodicity (Ruddiman and Raymo 1988). The peak of the last interglaciation was around 120,000 years ago, and the last glacial period, known in New Zealand as the Otira glaciation, extended from around 100,000 to 15,000 years ago. This itself consisted of periods of glacial maxima (stadials) interrupted by at least three short periods of warming (interstadials) (McGlone 1985). The most recent glacial stadial was 25,000 to 15,000 years ago, and is the one most likely to have left any imprint on current patterns of plant distribution in the South Island (McGlone 1985).

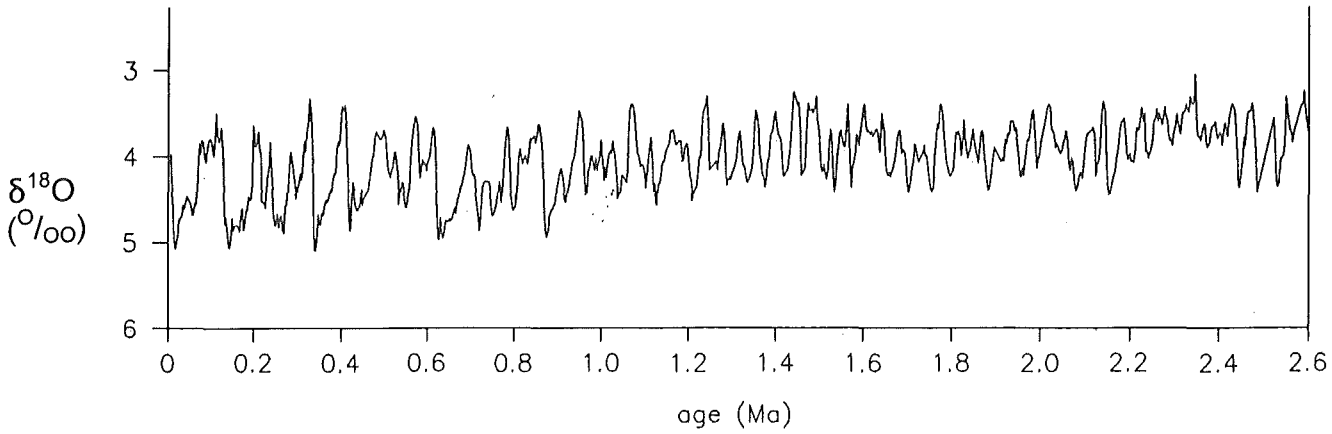

Fig. 3.1 Climate fluctuations of the last 2.6 Ma as depicted by benthonic oxygen isotope data for Ocean Drilling Program (ODP) Site 677. Reproduced from Shackleton et al. (1990)

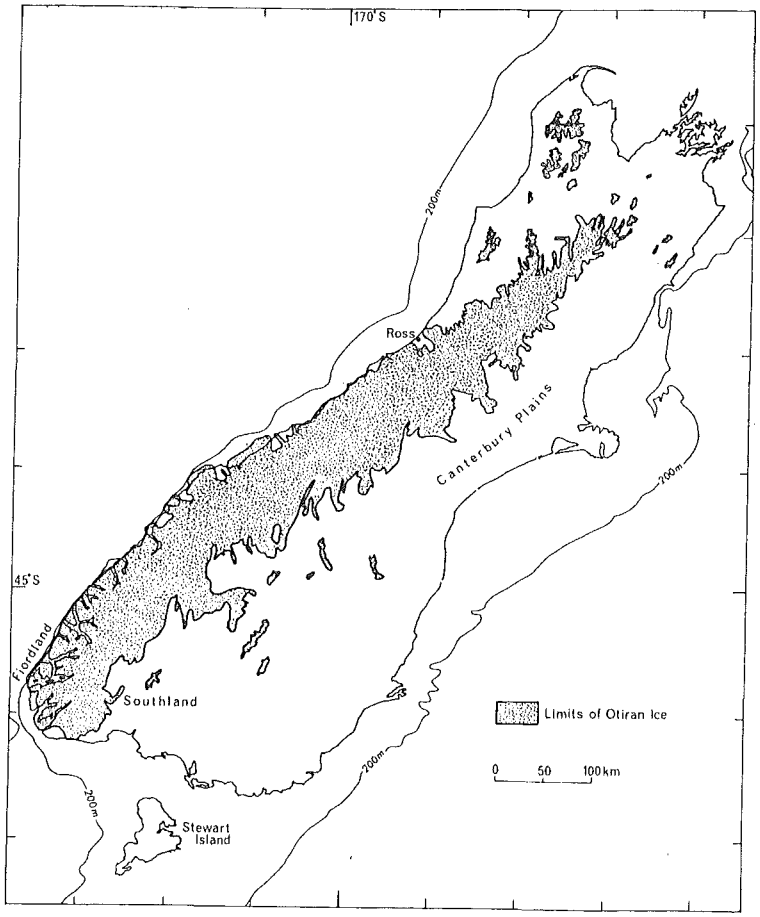

Fig. 3.2 The South Island showing distribution of ice during the height of the last glacial period (Otiran). Reproduced from Wilson (1978).

Under this hypothesis, it is very difficult to estimate how recently a continuous distribution existed for a taxon such as *Nothofagus*. One possibility is that during the Pleistocene, distributions have been cyclically continuous and disjunct, in step with interglacial and glacial periods respectively. In this case *Nothofagus* may have had a continuous distribution as recent as the most recent interglacial period, around 125,000 years ago. However if interglacial periods have not been long enough to allow closure of distributional gaps, then the most recent continuous distribution may date from earlier interglacial periods. A further possibility is that continuous distributions may have been preserved for *Nothofagus* during the less severe glacial periods prior to 0.9 mya, and only become disjunct in the intense glacial periods since then.

Early speculation was that *Nothofagus* was eliminated from the central region of the South Island as a direct result of continuous glacial ice cover during the last glacial period (Cockayne 1926; Willett 1950; Holloway 1954). This explanation is not feasible, given, for example, that *Nothofagus* is now abundant in Fiordland, which was heavily glaciated (McGlone 1985).

The generalised hypothesis of Wardle (1963) and Burrows (1965) attributed the chief cause for the South Island disjunction to Pleistocene climate, emphasising the role of general climatic and edaphic conditions, as well as the extent of ice advance, in accounting for current patterns of disjunction and endemism. The occurrence of endemic and disjunct plant species in Nelson-Marlborough and Otago-Southland was accounted for by a higher rate of species survival in these regions during the glacial periods (Wardle 1963; Burrows 1965). This was explained by a wider variety of available habitats prevailing in the north and south compared to the narrow 'waist' of the island. Surviving taxa persisted in refugia where conditions were conducive to their survival, and refugia subsequently served as centres of dispersal for outward migration during the Holocene. *Nothofagus* was believed to follow this general pattern; in the far south it was thought to survive in refugia in sheltered coastal locations (Wardle 1963).

The hypothesis holds that historical factors, rather than ecological factors, are the main reason for disjunctions. This implies that taxa may still be recovering their

potential ranges following the last glacial period, and therefore that their distributions are not stable.

3.1.2 'Environmental barriers' hypothesis

This hypothesis contends that the primary factors in causing and maintaining disjunctions are environmental, restricting the distribution of many species. In the case of *Nothofagus*, this is a combination of edaphic and climatic factors, combined with barriers to effective dispersal (McGlone 1985; McGlone et al. 1996).

The environmental conditions restricting *Nothofagus* from the central region of the South Island may be ultimately caused by the modification to the central South Island landscape by late Cenozoic tectonic activity (McGlone 1985). Uplift of the Southern Alps commenced about 10 million years ago, when movement became compressional at the boundary between the Australia and Pacific plates (Kamp 1992). The effects of this were most pronounced adjacent to the Alpine Fault, a 340 km length of the plate boundary in the central and northern South Island, where major deformation and vertical uplift occurred (Kamp 1992). Uplift has accelerated substantially in the last 5 million years (Walcott 1979, 1998).

The central portion of the South Island, particularly near the Alpine Fault, has experienced the greatest extent of uplift and erosion (Fig. 3.3), with estimates of a total of about 20 km of uplift occurring (Tippett and Kamp 1993). After soft Cenozoic rock was eroded away about 4-5 million years ago, upland topography comprising harder rocks came into being (Tippett and Kamp 1995a, 1995b). Aided by rapid uplift and high precipitation, denudation continues at a rate comparable to that of rock uplift (Wellman 1979; Kamp and Tippett 1993). Consequently, surfaces, particularly those on the western slopes of the Southern Alps, are very unstable, and soils tend to be eroded away within 2,000 years of formation (Tonkin and Basher 1990). Sediment yields in rivers draining the area are extreme by world standards, and there is a high rate of deposition on lowlands, particularly to the west of the ranges (Whitehouse and Pearce 1992).

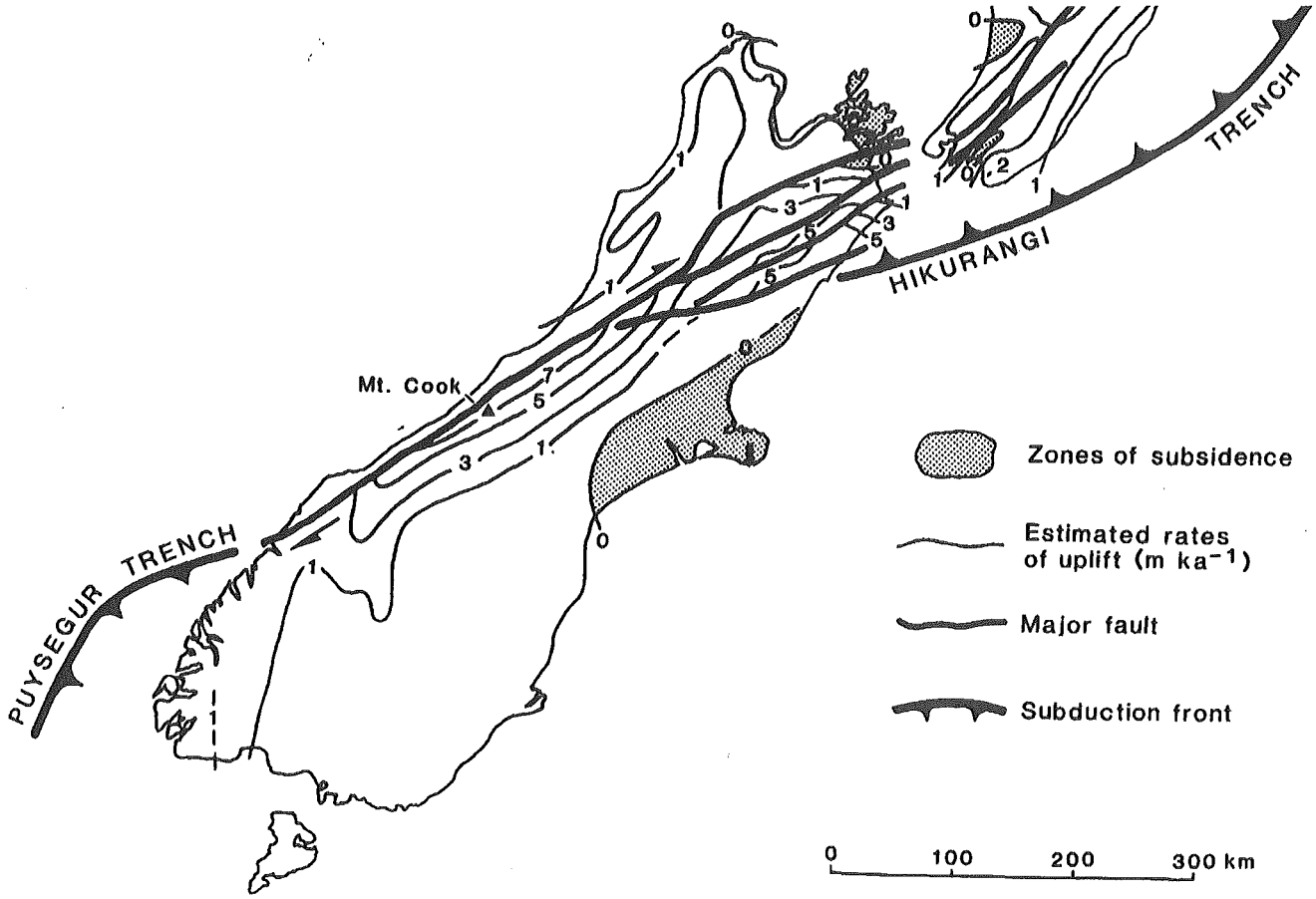

Fig 3.3 Current uplift rates in the South Island. Highest rates are in the central region of the Southern Alps.

Reproduced from Williams (1988).

The resulting edaphic conditions, in tandem with climatic factors, exclude *Nothofagus* from the central region during this interglacial, and possibly earlier interglacial periods. On the western side of the Southern Alps very high rainfall is induced by uplift of moist westerly air over the mountain ranges. Conversely to the east, fohn winds maintain a dry climatic regime. In these conditions *Nothofagus* is believed to compete poorly against better-adapted taxa (McGlone 1985; McGlone et al. 1996).

As for the glacial refugia hypothesis, it is difficult to estimate how recently a continuous distribution existed for *Nothofagus*. This depends on how recently environmental conditions in the gap last gave *Nothofagus* a competitive advantage over other vegetation groups. If recent glacial and interglacial periods had similar climatic regimes to the current one, *Nothofagus* may have been disjunct over the latter half of the Pleistocene at least. Changes in edaphic conditions as a result of tectonic activity would also have been a factor. For example, *Nothofagus* may have had a continuous distribution until intensification of tectonic processes in the last 1-2 million years (Walcott 1998) changed edaphic regimes in the central portion of the South Island.

The environmental barriers hypothesis predicts that distributions (including disjunctions) for most plant taxa are older and more stable than they would be under the glacial refugia hypothesis (McGlone 1985), since they are based on long-term geological events as well as rapidly oscillating climatic events. Most plants currently found in a particular region have been present in that region (even if limited to small refugia) during recent glacial and interglacial cycles at the very least (McGlone 1985). In regard to *Nothofagus*, this hypothesis can therefore be set apart from the glacial refugia hypothesis on the basis of a number of predictions about the current stability of distributions, number and extent of glacial refugia and speed of migration (Section 3.4).

3.1.3 'Lateral plate shift' hypothesis

This hypothesis states that the disjunctions have been created from once contiguous populations being split and rafted apart by 480 km of lateral movement along the Alpine Fault (Heads 1998). Heads (1998) implicates *Nothofagus* as a taxon whose

disjunction could have arisen in this fashion. This hypothesis predicts that the disjunct distributions of taxa whose disjunction arose in this way would be limited to opposite sides of the Alpine Fault.

The original timing of the start of lateral movement along the plate boundary appears to have been around 25 million years ago (Smale 1991; Kamp 1992). Therefore the hypothesis predicts that populations rafted apart by plate movement have become genetically isolated over time spans in excess of 20 million years.

In addition to objections to the general hypothesis, notably that it was advanced solely on the basis of distribution maps (Wallis and Trewick 2001), its relevance to *Nothofagus* appears weak. The hypothesis depends on disruptions to current distributions of taxa when they cross the Alpine Fault. However current distributions of *Nothofagus* do not support the predictions of this hypothesis. Distributions cross the fault with no apparent disruption. Almost without exception the four species exist on both sides of the Alpine Fault, both north and south of the beech gap. The one exception is *N. truncata*, due to its very limited distribution to the south, yet this species occurs on the same (west) side of the Alpine Fault on each side of the beech gap.

3.2 Hypotheses for disjunction due to dispersal

In contrast to the three vicariance hypotheses (Section 3.1) is the long-distance dispersal hypothesis. This proposes that the disjunction is caused by long distance dispersal of *Nothofagus* propagules across the central portion of the South Island. This scenario envisages the former existence of *Nothofagus* in the north of the island only, and the absence of some or all species in the south; for example the extirpation of the less hardy *N. fusca* and *N. truncata* from the south during glacial periods of the Pleistocene. In terms of long-distance dispersal of seed, the scenario has found no support (Wardle 1963; Wardle 1988; McGlone et al. 1996).

The hypothesis tends to be rejected on the grounds that beech seed disperses only short distances (Wardle 1963). The distance of seed dispersal (based on current distributions) would have been at least 75 km for *N. solandri* var. *cliffortioides* and *N.*

menziesii, and around 250 km for *N. fusca* and *N. truncata*. However evidence is emerging that *Nothofagus* seed can disperse greater distances than previously thought. It appears to regularly disperse at least several kilometres in strong winds (Section 1.1). The location of outlying stands of *Nothofagus* at the boundaries of the beech gap has led researchers to conclude that *Nothofagus* has dispersed distances of 6km (Wardle 1980) and 12 km (June 1982) onto receptive open habitats, although the assumption is that these are not relict populations, and that there were no former bridging stands. The ability of *Nothofagus* to disperse intercontinentally has been rethought over the last decade. Pollen and DNA evidence now suggests that *Nothofagus* seed dispersed between Australia, New Zealand and South America after these landmasses had reached present-day distances (ie thousands of kilometres) between each other (Hill and Dettman 1996). These events may have occurred in the order of millions, if not tens of millions of years ago. On the other hand post-glacial seed dispersal across the beech gap, although a much shorter distance, has had a much shorter time frame (by a factor of up to a thousand) in which to occur.

A further argument against the long-distance seed dispersal hypothesis is whether *Nothofagus* should have established in suitable habitats at intermediate distances within the disjunction. This would not necessarily be the case. The environmental barriers hypothesis predicts that habitat suitable for *Nothofagus* does not exist within the disjunction, unless perhaps in relatively small patches. In Westland for example, it is questionable whether any environment exists which offers *Nothofagus* a competitive advantage against podocarp / broadleaf mixtures. To the east of the Southern Alps, where the disjunction is mostly shrubland and grassland habitat, conditions may be suitable for *N. solandri* var. *cliffortioides*. However conditions may be too dry and cold for *N. fusca* and *N. truncata* to establish.

Consequently, for *N. fusca* and *N. truncata* at least, the possibility that the disjunction is caused by long distance seed dispersal should not be neglected.

A second dispersal hypothesis is that of long-distance dispersal of *N. fusca* and *N. truncata* pollen from the north to the south. This envisages the hybridisation with *N. solandri* var. *cliffortioides* in the south, with introgression over many generations leading to 'reconstitution' of these species (Wardle et al. 1988) (Section 3.5).

3.3 *Nothofagus* during the Pleistocene glacial periods

Much of the debate among the vicariance hypotheses, and between vicariance and dispersal hypotheses, centre on the survival of *Nothofagus* in the South Island during the glacial periods. Ice covered large areas, and elsewhere climates were severe. To the south of the present-day disjunction, in particular, there is disagreement over whether all *Nothofagus* species survived. The only glacial period for which there is much evidence for climatic conditions and vegetation cover is the most recent (Otiran), although seafloor ^{18}O patterns suggest that earlier glacial periods over the last million years had similar conditions (Fig. 3.1).

3.3.1 Glaciation during the glacial periods

Exclusion of vegetation by direct ice cover occurred over a large part of the South Island. A large complex glacier system consisting of expanded valley and piedmont glaciers extended 700 km along the Southern Alps, and averaged 100 km in width (Fig. 3.2) (Porter 1975). Where unimpeded by topography, such as on the coastal lowlands to the west, glaciers spread laterally and coalesced into continuous piedmont ice sheets with individual lobes up to 50 km wide (Porter 1975). These reached to about and below present sea level on the West Coast (Gage and Suggate 1958).

To the east, glaciation extended well down valleys past the ends of present-day lakes such as Pukaki, Ohau and Hawea, and through the mountain front overlooking the Canterbury Plains (New Zealand Geological Survey 1973). The ranges east of the Main Divide carried valley and cirque glaciers, but glacial activity decreased with increasing distance eastwards (Porter 1975; Soons 1979).

3.3.2 Climate during the glacial periods

Regional snowlines are postulated to have been around 800-850 metres lower than at present (Porter 1975; Soons 1979), suggesting that mean annual temperatures in the South Island were around 4-5°C lower (Soons 1979).

However, pollen evidence suggests that conditions were more severe than the simple temperature decrease would suggest (Wardle 1988). In the southern hemisphere, mean wind speeds may have been higher, possibly 1.5-1.8 times greater than at present (Bowler 1979; Petit et al. 1981), with winds from the west to north-west predominating (Bowler 1978). Additionally, conditions may have been drier, since cooler ocean temperatures during glacial maxima may have meant less moisture carried by airstreams (McGlone 1988). Although there is little proof of lower precipitation in New Zealand (McGlone 1988), there is some evidence for much drier regimes in Australia during the latter stages of the last glacial period (Bowler 1978).

Stronger, more westerly, winds would have accentuated the steep precipitation gradient from west to east across the South Island, and increased the likelihood of generally drier conditions and prolonged drought to the east of the main divide (McGlone 1988). These conditions could have exacerbated the dryness of the late Pleistocene coarse gravel surfaces of the Canterbury plains, which, given the absence of sheltered sites, was unsuitable for forest trees (Soons 1979).

Several authors have suggested that as a result of the northward shift of the cold water of the Antarctic convergence, New Zealand would have been subjected to episodes of intense maritime polar airmasses (Soons 1979; McGlone 1988).

3.3.3 *Nothofagus* survival during the last glacial period

Our knowledge of *Nothofagus* distribution in the South Island during the last glacial period is imprecise, due to a paucity of good quality evidence. The strongest proof of *Nothofagus* survival would be macrofossils, but there is no known record of *Nothofagus* macrofossils dating from the last glacial period anywhere in the South Island (M. McGlone pers. comm.).

The main source of evidence comes from fossil pollen in pollen cores, although these do not provide an accurate picture for *Nothofagus*. Being anemophilous, *Fuscospora* in particular has very high pollen production and its pollen is widely dispersed (McGlone 1988; McGlone et al. 1996). Thus it can be greatly over-represented in pollen samples where it is present, and appears in pollen samples in locations (such as the Chatham Islands, 800 km east of the South Island) where the tree is entirely absent

(McGlone 1988). Thus, low levels of *Fuscospora* pollen in a core do not necessarily imply that *Fuscospora* was present in the general region, let alone locally (McGlone 1988). *N. menziesii*, on the other hand, is under-represented in the pollen rain, and its presence in cores are a far stronger indicator of local presence (McGlone et al. 1996).

Due to the great difficulty in distinguishing *Fuscospora* fossil pollen at the species level (M. McGlone pers. comm.), pollen cores reveal little about which individual *Fuscospora* species survived in the area. Therefore, even though pollen evidence suggests that *Fuscospora* survived on the southern side of the modern-day disjunction, just which of the individual species did so can not be resolved.

Pollen core analysis suggests that *Nothofagus* was the most common forest tree in the South Island. However, given its low proportion (<10%) in overall pollen counts, its distribution was generally sparse, while grassland and shrubland dominated throughout (McGlone 1985, 1988). Very low counts suggest that *Nothofagus* was completely absent in some regions, particularly in inland and eastern areas of the island (Moar 1980; McGlone 1988). On the West Coast however, higher levels of both *Fuscospora* and *N. menziesii* pollen suggest a greater presence than in the east (McGlone 1985). There is particularly good evidence for persistence of relict populations in northern parts of the region, for example between the Grey and Taramakau rivers (Moar and Suggate 1996).

There is little concrete evidence for forest tree survival to the south of the modern-day disjunction, let alone evidence for any local distribution patterns. Pollen cores indicate dominance of grassland and shrubland. The almost ubiquitous low *Fuscospora* pollen component can be explained by long-distance dispersal from the north, and not necessarily by local dispersal. The general agreement that at least some *Fuscospora* taxa survived in the far south during the glacial periods tends to be based on their current distributions and environmental tolerances (Wardle 1963; Burrows 1965; McGlone 1985).

As already indicated (Section 3.3.2), the apparent scarcity of *Nothofagus* forest cannot be explained solely by the lowering of temperature by 4-5°C (McGlone 1985, 1988). *Nothofagus solandri* var. *cliffortioides* and *N. menziesii* currently form timberlines at the average 10°C January isotherm. On this basis, timberlines during the glacial

periods would have been around 500m in the central South Island and 200-250m in the south of the island (McGlone 1988), and would imply wide areas of continuous forest cover.

Thus other factors appear to have limited the distribution of *Nothofagus* and other forest trees during the glacial maximum. A key factor may have been episodes of polar airmasses across New Zealand (Section 3.3.2). Given that *N. solandri* var. *cliffortioides* and *N. menziesii* are seriously damaged at air temperatures below -10°C, frosts accompanying cold airmasses may have eliminated, and prevented the re-establishment, of large areas of forest (McGlone 1988). Strong winds accompanying these cold episodes may have restricted exposed forest in favour of low-growing shrub communities (McGlone 1988).

The very low *Nothofagus* pollen counts in the east of the South Island are not surprising, given that forest development was probably inhibited by dry, often droughty, conditions, accompanied by an increased incidence in fire (McGlone 1988). The winter snowline (and hence upper limit of tree growth) was at the level of the inner edge of the Canterbury Plains, and thus the alpine scrubland and grassland zone could have come right down to the plains (Soons 1979). The plains themselves were also probably unsuitable for trees (Section 3.3.2).

3.4 'Glacial refugia' and 'environmental barriers' hypotheses compared in respect to *Nothofagus*

Most of the debate over the major South Island disjunctions, and the related question of distribution of endemics, has been between the two camps representing the glacial refugia and environmental barriers arguments. Due to the importance of the *Nothofagus* disjunction (Section 1), *Nothofagus* has been at the heart of this debate, and a great deal of research effort, including palynological studies, population structure and migration patterns, have been devoted to the genus.

Clearly, there are other possible reasons why *Nothofagus* is absent from the South Island gap, as is evidenced by other areas in New Zealand (eg Mt Taranaki, Manawatu Gorge area) which didn't have continuous ice cover or abnormally severe

glacial climates (McGlone 1985), and which have not been subject to recent major geological change.

The opposing hypotheses bring with them a number of opposing predictions (Sections 3.4.1 - 3.4.4).

3.4.1 Migration of *Nothofagus* into areas suited to it

In the glacial refugia hypothesis, refugia became centres of dispersal after the most recent glacial period ended. Plant species migrated outwards and back into regions suitable for them, although under constraints of availability of suitable environments, dispersal strategies, migration routes and available soil profiles (Wardle 1963; Burrows 1965). The hypothesis contends that in many cases full adjustment to current climatic conditions have yet to be attained (Burrows 1965), and that many species have yet to regain their former distributions (McGlone 1985). This would predict therefore, that the current distributions of such species are often not constrained by environmental factors, but instead by the rate of migration into areas suited to them, and which they occupied prior to the glacial maxima. This prediction has been applied to the *Nothofagus* gap in Westland, insofar as the genus has failed to yet occupy areas "that appear suited to it" (Wardle 1963), and that it is in the process of reclaiming the gap from both north and south (June 1982; Wardle 1988).

Conversely, the environmental barriers hypothesis predicts that since plant distributions are older and relatively stable, environmental barriers are the main factor constraining the distribution of species (McGlone 1985). These will often be manifested through the inability to compete with better adapted plants (McGlone 1985). Thus, in the case of *Nothofagus*, the broad distribution of the genus is assumed to be more or less static. Current range shifts are due to short-term fluctuations in Holocene climate as opposed to long-term migration back from Pleistocene refugia (McGlone 1988). Wardle (1988), in opposing this view, argues that there are many environments, including within distribution gaps, which are suited to but not currently occupied by disjunct species.

Consequently, the question of whether there is current-day expansion of the range of *Nothofagus* at the boundaries of the beech gap has been seen as important by some

(Wardle 1980; June 1982) who view expansion as part of the overall picture of reclamation of pre-glacial distributions by *Nothofagus*. McGlone (1985) however rejects the value of such studies, on the basis that population dynamics at boundaries are a reflection of present-day climatic regimes only, and not of long term climatic fluctuations since the last glacial maxima. The authors of two studies however contend that long-term reclamation of the gap is occuring. Wardle (1980) concluded from palynological evidence and current distribution patterns that *N. menziesii* is currently spreading north into the southern edge of the gap. June (1982) proposes on the basis of population structures of *Nothofagus* patches, a boundary advancing southwards at the northern edge of the gap. McGlone (1985) concludes that Wardle's (1980) findings could be interpreted as equally supportive of the opposing view, that environmental factors are limiting range expansion northwards. In both studies, he considers that although the pollen evidence suggests increased *Nothofagus* prominence in the respective areas for thousands of years, it does not point to range expansion (McGlone 1985).

However, even if the boundary were more or less static, given fluctuating Holocene climates, it would still be difficult to reject the glacial hypothesis on this basis. It is likely, as Wardle (1988) points out, that a discontinuity in suitable habitats could slow, or arrest, migration of a plant species. For *Nothofagus*, the confinement of the migrational route between the Southern Alps and the Tasman Sea means that migrational pathways may be blocked (temporarily at least) by interceding areas where *Nothofagus* seedlings are uncompetitive (Wardle 1988).

Ultimately there is a fine line between arguing for a discontinuity of suitable habitats of migration routes for *Nothofagus*, or on the other hand arguing that suitable habitats are so scarce that the region as a whole represents an environment unsuitable for *Nothofagus*. *Nothofagus* has been present in South Westland for a long time, yet is still confined to scattered sites and is only really dominant in the mountain valleys.

3.4.2 Distribution and number of refugia

The glacial hypothesis presumes that plant distributions of many plant taxa were very restricted during the glacial maxima, and that current distributions can be explained

by long-distance migration over the Holocene. Thus few forest tree species survived, and even these in very limited coastal sites (Wardle 1988). A corollary of this hypothesis is that forest species, including *Nothofagus*, can migrate rapidly under favourable conditions, despite putative low dispersal distances (Section 3.4.3).

On the other hand the environmental barriers hypothesis, in advocating long-term stability in overall distributions, predicts that tree species with a distribution in a region have been present there over a long time span, spanning several of the most recent glacial and interglacial cycles at the very least (McGlone 1985). Glacial maxima did not cause their elimination from the region, but rather a retreat to small localised refugia with suitable microclimates (McGlone 1985, 1988). This implies that, in the far south, *Nothofagus* was present in widely dispersed coastal and inland sites in Otago, Southland and south Westland. Therefore the general assumption that *Nothofagus* only migrates slowly will still account for its current distribution (McGlone and Bathgate 1983) (Section 3.4.3).

There is still no convincing proof that any of the *Fuscospora* species survived on the southern side of the modern-day disjunction (Section 3.3.3). If *Fuscospora* did survive there, uncertainty over the intensity of climatic factors (Section 3.3.2) means that the number and location of suitable refugia can only be conjecture. Adherents of the environmental barriers hypothesis, favouring survival of *Nothofagus* in a number of widespread sites, tend to be generous in their appraisal of suitable sites. They argue that environments conducive to forest growth would have been low to mid-slope on warm north-facing slopes in hilly country (McGlone 1988), even some distance inland (McGlone and Bathgate 1983). These locations would have been less exposed to climatic extremes through often being cloudier and wetter, sheltered from prevailing winds, and subject to less severe frosts than adjacent valley floors (McGlone and Bathgate 1983; McGlone 1985). Survival of a number of forest taxa (including *Nothofagus*) has been inferred from pollen evidence of abrupt regional reafforestation in the south in the Holocene (McGlone 1988).

On the other hand, proponents of the glacial hypothesis, favouring highly restricted tree distributions during the glacial maxima, are more conservative in their assessment of sites suitable for forest trees. Wardle (1988) argues, using pollen evidence for support, that the only forest trees hardy enough to survive glacial maxima conditions

anywhere in the south were *N. solandri* var. *cliffortioides* and *N. menziesii*. Even these two species could only have persisted in small patches near sea level in the mildest coastal sites (Wardle 1963; Burrows 1965; Wardle 1988). Suggested suitable areas have included the south-east coast of Otago (Wardle 1963) and the Arawata and Paringa-Moeraki districts in South Westland (Wardle 1980), where elevated unglaciated terrain near the coast may have provided sites.

The environmental barriers and glacial refugia hypotheses also make different predictions about the survival of *Nothofagus* in inland Canterbury, to the east of the Main Divide. Both *N. solandri* var. *cliffortioides* and *N. menziesii* are found, in patches at least, throughout most of this region (Section 2.3.2). The environmental barriers hypothesis should predict persistence of *Nothofagus* in these areas, whilst the glacial refugia hypothesis predicts elimination from the whole region and subsequent migration from the north. Although low pollen counts are not reliable, the virtual absence of tree pollen dating from the last glacial period (Section 3.3.3) would appear to support the latter hypothesis.

In an argument where good quality evidence in the form of fossil data is lacking, the greater burden of establishing proof would appear to lie with adherents of the environmental barriers hypothesis. Their task is to provide concrete proof of presence of *Nothofagus* (and other tree species) at a wide range of sites during the glacial maxima. The adherents of the glacial hypothesis, on the other hand, have some justification in arguing for the widespread absence of tree taxa unless proven otherwise.

3.4.3 Migration rates of *Nothofagus*

Arguments relating to the proposed locations of refugia during glacial maxima, are bound up with questions relating to the migration rate of advancing tree species, and this is nowhere more so than with *Nothofagus*, often believed to advance slowly on account of the low dispersal distance of its seed.

The glacial hypothesis, in presuming that *Nothofagus* had a very restricted distribution during the last glacial period, must explain the current widespead distribution of this taxa south of the disjunction in terms of migration across long distances during the

Holocene. Conversely the environmental barriers hypothesis, in predicting a more widespread distribution of *Nothofagus* during the last glacial period, can more easily accommodate conventional assumptions about the short dispersal distance of the genus.

Adherents of the glacial hypothesis, therefore have the task of proving that advancement of beech forest fronts occurred more quickly than its short-distance seed dispersal would suggest. Migration of stands of beech through existing tall, dense vegetation is generally considered extremely slow (Wardle 1988), although there is some evidence that the availability of open habitats can permit rapid migration. This may have been the case in the Landsborough valley, which was glaciated in its entire length during the last glacial maxima. For *N. menziesii* to have spread to the head of the valley during the post-glacial period would have required migration of at least 700m per century (Wardle 1980). There is some evidence that *Nothofagus* seed can frequently disperse at least several kilometres (Section 3.2). Rapid advance of *Nothofagus* could thus be accounted for by seed dispersal across many kilometres into favourable disturbed sites or sites in heathland, which in turn become the nuclei for (slow) outward spread (Wardle 1980; June 1982).

3.4.4 *Nothofagus* in the beech gap during previous interglacial periods

The glacial refugia hypothesis predicts that progressive encroachment into the gap during interglacial periods would, if the interglacial lasted long enough, result in substantial (if not complete) closure of the gap. The hypothesis would thus be assisted by the discovery of *Nothofagus* evidence well inside the gap dating from previous interglacials.

There is no evidence (from pollen sequences) of *Nothofagus* having survived far inside the confines of the beech gap in the last (Oturan or Kaihinu) interglacial (Wardle 1988). However there is both *Fuscospora* and *N. menziesii* pollen evidence, considered to date from the late cooling phase of the interglacial, from sites up to 20 km south (to around Hokitika) of the current northern edge of the gap (Moar and Suggate 1996). This suggests some range expansion of *Nothofagus* forest into the gap

at that time, but this could be explained as range variation as a result of normal interglacial climate fluctuations, and not as part of a long term advance.

3.5 Survival of *Nothofagus fusca* and *N. truncata* in the far south

It is possible that the central South Island disjunction can be attributed to different causes for the different *Nothofagus* species. There is general consensus that *N. solandri* var. *cliffortioides* and *N. menziesii* were hardy enough to survive in the south of the South Island during glacial periods (Wardle 1963; Burrows 1965; McGlone 1985), and that the disjunction in these species can adequately be explained by vicariance hypotheses. However the poorer cold tolerance of *Nothofagus fusca* and *N. truncata* brings into question whether they were able to survive in the south of the South Island. If they did not survive, their current distribution can only be explained by hypotheses of long-distance dispersal during the Holocene.

At best *N. fusca* and *N. truncata* must have been very marginal in the far south. *N. fusca* in the south barely reaches 800 m in altitude, and even that only on warm, sheltered slopes. *N. truncata* is currently known only from stands no higher than 100 m on coastal hills in South Westland (June 1977), and even north of the disjunction its current altitudinal limit is only 750m. On the basis that snowlines, and therefore vegetational belts, were lowered by 800-850 m during the last glacial periods, Wardle (1988) doubts whether either species could have survived. In fact, further doubts can be cast given that *Nothofagus* may have been even more adversely affected than is suggested by temperature factors alone (Sections 3.3.2 and 3.3.3).

In *Fuscospora*, if one assumes that *N. solandri* var. *cliffortioides* survived in the south of the island during the last glacial period, then three alternative hypotheses explain the current distributions of *N. fusca* and *N. truncata*.

1) *N. fusca* and/or *N. truncata* survived in the south along with *N. solandri* var. *cliffortioides* during the last glacial period.

2) *N. fusca* and/or *N. truncata* failed to survive in the south during the last glacial period, and have since re-established there via long-distance seed dispersal (discussed in Section 3.2).

3) *N. fusca* and/or *N. truncata* failed to survive in the south during the last glacial period, and have since re-established there via long-distance pollen dispersal and hybridisation. In this scenario *N. fusca* and *N. truncata* pollen dispersed from the north to the south of the disjunction, where hybridisation with resident *N. solandri* var. *cliffortioides* occurred. Successive generations would have seen (i) backcrosses to the pollen parent or (ii) crosses with other hybrids with selection favouring phenotypes resembling the pollen parent. In habitats favouring the distant pollen parent, this would ultimately have led to the 'reconstitution' of this species (Wardle et al. 1988).

In the only previous molecular study of this question, Haase (1992) rejected the two dispersal options (2 and 3) in respect of *N. truncata*, on the basis of isozyme analysis. A population of *N. truncata* from south Westland differed substantially from northern South Island populations. They had a reduced genetic diversity having fixed alleles in most loci, whilst in the one variable locus allele frequencies varied markedly from those further north. There were however no novel alleles in the southern population. Haase attributed the genetic divergence to genetic drift in small populations across a long time period. He concluded that *N. truncata* in south Westland had been present throughout the last glacial period at least, and did not migrate south across the disjunction during the Holocene.

However Haase failed to acknowledge the possibility that the observed genetic divergence could equally have arisen from relatively recent founder populations. Consequently Holocene long-distance dispersal can not be discounted on the basis of his results.

4 A phylogeographic approach using chloroplast DNA

Phylogeography is the study of the principles and processes governing the geographic distributions of geneological lineages, especially those within and among closely related species (Avise 1994, 2000). This discipline requires molecular markers which can be used for phylogenetic inferences, and which will reveal a degree of spatial differentiation in the target taxon. It can then be used to help re-create the history of the taxon in relation to its geographical setting. In this case, phylogeographic techniques are used to help explain how present-day distributions of *Fuscospora* have been influenced by historical climatic and geological processes.

4.1 Molecular evolution in biogeographical studies

Molecular techniques allow biogeographical studies to be based on the evolution of DNA. Central to this is the molecular clock, the idea that evolution of DNA is proportional to clock time (Clegg and Zurawski 1992). This does not imply, however, that the evolutionary rate of a given section of DNA is perfectly regular; changes occur randomly over time, and the process is best described as "stochastically constant" (Avise 1994). Nor can it be implied that evolutionary rates are uniform across all taxa. There is evidence of substantial rate heterogeneity in nuclear DNA, mtDNA (Hillis et al. 1996) and cpDNA (Bousquet et al. 1992). Consequently, when selecting genetic markers for a taxon with unknown evolutionary rate, it is important to select markers which have shown appropriate variation in closely related taxa.

Appropriate molecular markers are available for all levels of study, and permit the construction of phylogenies from the highest taxonomic levels to the population level. Phylogenetic analyses can then be used to test biogeographical hypotheses and evolutionary processes (Moritz and Hillis 1996). The wide range of sequence evolution rates across the genome mean that sequences appropriate to the problem at hand can be selected for study; evolving rapidly enough so that meaningful variation

can be measured, but not so much that excess variation confounds results (Dowling et al. 1996)

At the intraspecific level, closely related organelle haplotypes have often been geographically localised, whereas genetically distant haplotypes have reflected greater degrees of geographical separation (Avise 1994). This suggests that geographic distributions of species can be overlain with intraspecific phylogenies. The degree of organellar genetic distance can therefore be used to estimate the time of separation among fragmented populations, or across disjunctions. This integration of population-level phylogenetic analyses with biogeography has greatly increased the ability to infer past patterns of geographic distribution of species, gene flow and hybridisation processes (Soltis et al. 1992). Organelle genomes, with their normally uniparental inheritance, are particularly useful tools in this discipline (Section 4.3).

4.2 Evolution in the chloroplast genome

The chloroplast genome consists of a single circular chromosome (Downie and Palmer 1992) (Fig. 4.1). In angiosperms it is conserved in size and structure, ranging from 135 to 160 kb, and is characterised by an inverted repeat, two identical sequences of ~25 kb (Palmer 1987), which divide the remainder of the genome into a large copy region and a small copy region (Olmstead and Palmer 1994). Most angiosperm chloroplast genomes have a similar or identical arrangement of gene order, and major rearrangements are rare (Palmer 1987; Downie and Palmer 1992).

The rate of evolution in cpDNA is very slow compared with nuclear DNA (Palmer 1987; Soltis et al. 1992). For example the synonymous substitution rate in chloroplast genes appears to be less than half that of nuclear genes (Wolfe et al. 1987). On the other hand, rates of nucleotide substitutions are 3-4 times faster in the chloroplast genome than in the plant mitochondrial genome (Palmer 1992). Among plant lineages there is evidence of a high degree of rate heterogeneity in cpDNA evolution (Bousquet et al. 1992; Barraclough et al. 1996; Manen et al. 1998).

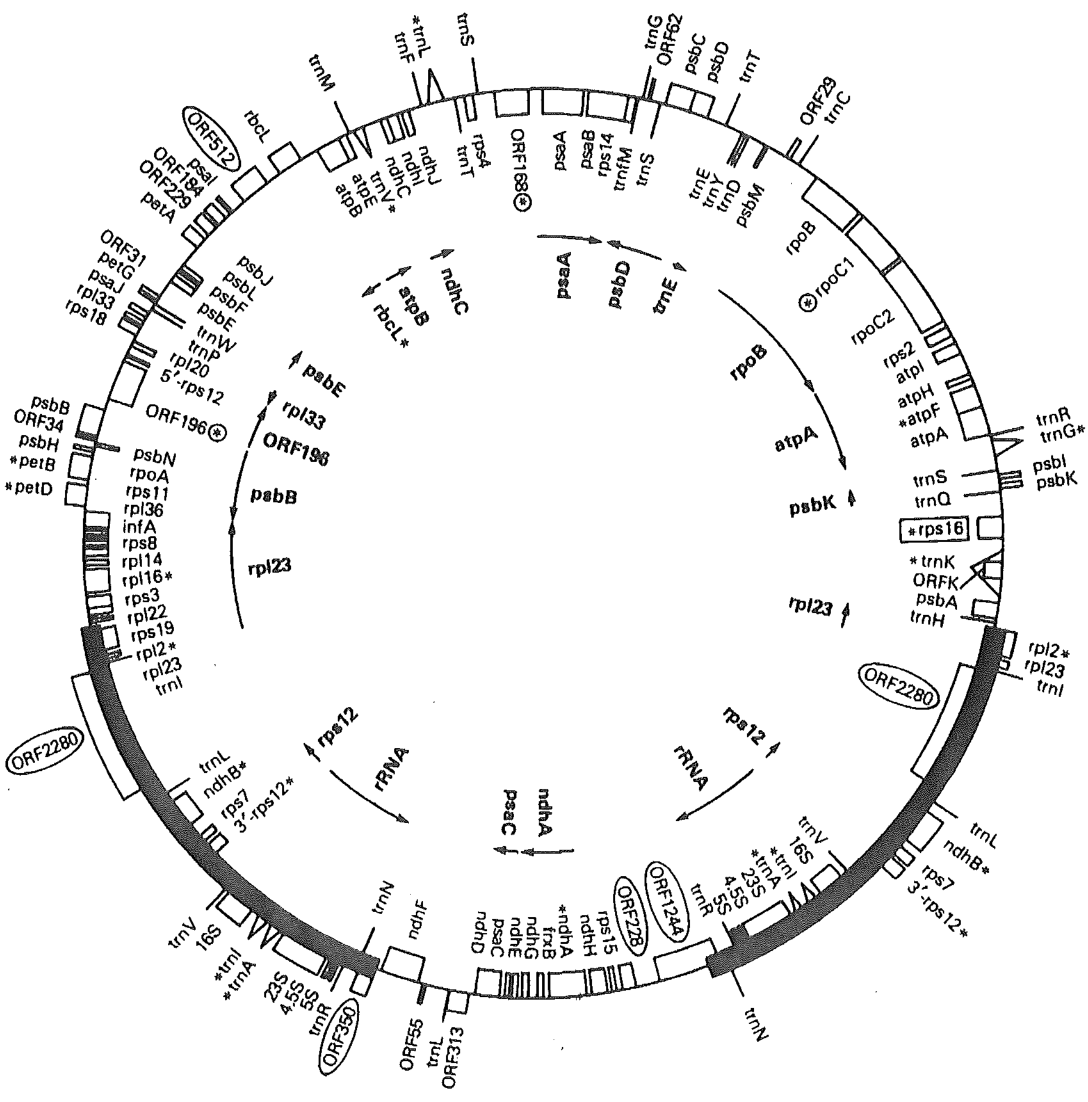

Fig. 4.1 Physical and gene map of the 156 kb *Nicotiana tabacum* chloroplast genome. Genes transcribed clockwise are shown on the inside of the circle, and those transcribed counterclockwise are shown on the outside. The thickened part of the circle denotes the inverted repeat.

Reproduced from Downie and Palmer (1992).

Evolution in non-coding intergenic spacer regions and introns tend to result from the accumulation of point mutations (Downie and Palmer 1992). These regions evolve more rapidly than the coding regions since they are not under selective constraint. For example, across a range of taxa, the non-coding *trn*L-*trn*F spacer and *trn*L intron were found to evolve more than three times faster on average than the *rbc*L gene (Clegg and Zurawski 1992; Gielly and Taberlet, 1994).

The chloroplast genome is usually uniparentally inherited, and in most angiosperms is via the maternal parent (Palmer 1987). The main reason for this is that the generative cell in pollen usually receives no chloroplasts, although there are other contributing factors (Mogensen 1996). However the nature and degree of uniparental inheritance is still not fully understood, and the exact mode of plastid inheritance is well-established in relatively few species (Mogensen 1996). Additionally, where plastid inheritance has been determined, inheritance is not always fully maternal. Paternal leakage arising from biparental inheritance has been observed, at least occasionally, in one-third of angiosperm genera studied to date (Mogensen 1996). Plastid DNA was detected in the pollen of 18% of angiosperm species screened, and most of these corroborate with genetic evidence of biparental inheritance (Corriveau & Coleman 1988).

4.3 Why chloroplast DNA is chosen for this study

The chloroplast genome is proving to be a particularly useful tool in the discipline of phylogeography (Section 4.1), and has assisted in the reconstruction of forest tree distributions at the last Pleistocene glacial maximum (Ferris et al. 1993; Dumolin-Lapègue et al. 1997b; Marchelli et al. 1998; McKinnon et al. 2001; Okaura and Harada 2002), and of the migration and gene flow within and between congeneric tree species during the Holocene (Whittemore and Schaal 1991; Soltis et al. 1992; Ferris et al. 1993; Dumolin-Lapègue et al. 1997b; Petit et al. 1997; Jackson et al. 1999; King and Ferris 2000; Okaura and Harada 2002).

The assumption is made in this study that cpDNA in *Nothofagus* is maternally inherited. However it is acknowledged that no study of the patterns of plastid

inheritance in *Nothofagus* has been made. The assumption of maternal inheritance is based on two facts. Firstly, the great majority of angiosperms are maternally inherited. Secondly *Fagus*, the nearest taxon to *Nothofagus* for which the mode of cpDNA inheritance has been demonstrated, has been observed to have at least predominantly maternal inheritance (Dumolin et al. 1995).

cpDNA is best suited for this study for the following reasons:

1) Maternal inheritance of cpDNA (Section 4.2) implies that gene flow of cpDNA is mediated by seed (Dumolin-Lapègue et al. 1997b). For species such as *Nothofagus*, where seed usually disperses short distances, this means that gene flow across larger distances is restricted or slowed. This is therefore more likely to result in cpDNA variation being geographically localised. This makes it very useful for phylogeographic studies, and provides valuable information for the inference of historical distributions and migration routes (Dumolin-Lapègue et al. 1997b).

2) Maternal inheritance may help trace the long-term effects of hybridisation in natural populations. This is because cpDNA may reveal introgression which may not be apparent from nuclear DNA data alone (Palmer 1987; Whittemore and Schaal 1991). The downside of this for phylogeneticists is that cpDNA-based phylogenies for currently or formerly hybridising taxa may be misleading. If these phylogenies reflect cpDNA introgression, they may not be congruent with accepted species boundaries or nuclear-based phylogenies (Clegg and Zurawski 1992; Soltis et al. 1992).

3) Markers based on maternal inheritance can be used to test the long-distance pollen dispersal and hybridisation hypothesis of Wardle et al. (1988) (Section 3.5). This hypothesis predicts that *Nothofagus fusca* and *N. truncata* south of the disjunction have been reconstituted from maternal *N. solandri* var. *cliffortioides* individuals in the same area. Therefore the chloroplast genomes of *N. fusca* and *N. truncata* should be identical to those of local populations of *N. solandri* var. *cliffortioides*.

4) A range of evolutionary rates in different parts of the chloroplast genome permits the tailoring of appropriate genetic regions to this study. Non-coding areas such as intergenic spacers and introns are the best candidates. They exhibit extensive

intraspecific variation, even at a population level, and have been informative in intraspecific studies across a very broad range of plant groups (Soltis et al. 1992). On the other hand, genes in cpDNA evolve slowly and tend to be more suitable for constructing higher-level phylogenies. However the respective suitability of non-coding and coding cpDNA for a particular taxon depends on various factors including the rate of evolution in the lineage, and the timing of divergence (Olmstead and Palmer 1994).

5) Analyses via sequencing and restriction enzymes of cpDNA regions are useful since evolution in cpDNA is mainly by point mutations rather than major rearrangements (Section 4.2).

6) The use of cpDNA is facilitated by its relative abundance (Clegg and Zurawski 1992). With between 20 and 200 copies of the genome in each chloroplast, and multiple chloroplasts in each leaf cell, there are typically a few thousand chloroplast DNA molecules in a single leaf cell (Palmer 1987). Since most plants are homoplasmic for organelle genomes, all copies of cpDNA are usually genetically identical (Ouborg et al. 1999).

7) There is now an extensive background of molecular information on the chloroplast genome, and complete sequences are now available for a number of species (Clegg and Zurawski 1992).

For a phylogeographic approach to be adopted, molecular techniques which produce phylogenies are required. These techniques require the comparison of homologous characters, derived from a common ancestor (Avise 1994, 2000; Swofford et al. 1996). Two of the most frequently used techniques, which are appropriate to this study, are sequencing (Section 4.4) and restriction fragment length polymorphisms (Section 4.5).

4.4 The use of sequencing techniques

The direct sequencing of nucleotide pairs in nucleic acids is a very powerful technique, since nucleotides are the basic units of information encoded in organisms; thus a very high resolution is offered (Hillis et al. 1996). It has become one of the

most utilised molecular approaches for inferring phylogenetic history (Hillis et al. 1996). Even Palmer (1987), who questioned the applicability of the slowly evolving chloroplast genome to intraspecific and populational questions, suggested that improvements (since realised) in DNA sequencing techniques would greatly increase the use of the genome for such questions by allowing the comparison of longer sequences between more samples.

It is essential that sequences compared from different samples are homologous (Hillis et al. 1996; Swofford et al. 1996). The nucleotide base pair differences are represented as multistate character data (Swofford et al. 1996) which are phylogenetically informative. Using a number of such characters, genetic distances can be inferred, to form the basis of a phylogeny of the genetic region (Moritz and Hillis 1996).

Sequencing is, however, labour-intensive and costly per individual and per locus examined (Hillis et al. 1996). A clear trade-off exists, and therefore a balance must be sought, between the number of cpDNA regions on the one hand, and the number of individuals on the other hand.

4.5 The use of restriction fragment length polymorphisms (RFLPs)

An indirect assay for sequence variation involves a comparison of the number and size of fragments produced by the digestion of the DNA with restriction endonucleases (Dowling et al. 1996). Restriction endonucleases are enzymes that cleave DNA at a constant position within a specific recognition sequence which is usually 4-6 bp long. Cleavage sites for a particular enzyme can be created or eliminated by the presence of substitutions or small indels at the recognition sequence, and the net result will be a change in the number and size of fragments detected by electrophoresis. These variations are restriction fragment length polymorphisms (RFLPs) (Dowling et al. 1996), and the characters used for phylogenetic analysis are the variable restriction sites, rather than the restriction fragments (Moritz and Hillis 1996).

The use of RFLPs gives less resolution than sequencing, since only a fraction of the genetic region is directly assayed. However the technique is cost-effective where many individuals, or large portions of the genome, are being screened for specific changes in sequence, and can very effectively complement sequencing studies. This is because nucleotide sequences are the basis for restriction site maps, allowing the direct interpretation of fragment variation (Dowling et al. 1996).

5 Aims of this project

The objective of this project is to infer a phylogeographical pattern among the New Zealand species complex of *Fuscospora* in the South Island. The phylogeny will be based on a number of non-coding regions of chloroplast DNA, and will be drawn from sequence and RFLP data. The pattern of spatial differentiation among cpDNA haplotypes will be used to assess historical influences in *Fuscospora* distribution, and in particular to evaluate the various hypotheses for the South Island disjunction.

The genetic distance between populations north and south of the disjunction may help indicate the age of the disjunction. A large genetic distance between haplotypes north and south of the disjunction would suggest that maternal-line gene flow declined a long time ago due to an early origin to the disjunction. Conversely, small or no genetic distance could indicate a recent origin to the disjunction, either through disjunction occuring in a recent glacial period, or as a result of Holocene long-distance dispersal.

A project of this scope is unlikely to reveal sufficient polymorphisms to allow anything more than a very broad estimate to be drawn. Consequently, given the difficulty of predicting the age of the disjunction under the 'glacial refugia' and 'environmental barriers' hypotheses (Section 3.1), this project is not expected to help resolve between these. For example, it would be most unlikely to allow discrimination between the disjunction arising from environmental barriers as a result of uplift of the Southern Alps (which may have arisen anything up to 4-5 million years ago) or from the earliest glacial periods of the Pleistocene (1-2 million years ago). Similarly, a very close (or absence of) genetic distance would probably not allow discrimination between disjunction arising from the most recent glacial period (100,000 years ago) and Holocene dispersal (less than 10,000 years ago). However, even the most approximate estimate may be used to evaluate the 'lateral plate shift' hypothesis (Section 3.1.3), which predicts that genetic variation would reflect geographical separation occurring at least 20 million years ago (Wallis and Trewick 2001).

The hypothesis of long-distance pollen dispersal and hybridisation (Section 3.5) will be tested by comparing the genotypes of *Nothofagus fusca* and *N. truncata* south of the disjunction with that of nearby *N. solandri* var. *cliffortioides*. Identical genotypes will, however, not necessarily be proof of this hypothesis. In other studies (eg Whittemore and Schaal 1991; Steane et al. 1998), the sharing of chloroplast genotypes has been attributed to introgression of the chloroplast genotypes resulting from hybridisation among sympatric species. If this study reveals the sharing of chloroplast genotypes in *Fuscospora* elsewhere in its range, this would suggest that cpDNA introgression is prevalent within the subgenus.

6 Methods

Sampling was performed at a number of sites around the South Island. Laboratory work was carried out in the Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, and at Landcare Research, Lincoln.

Sampling and testing was carried out on all three *Fuscospora* species in New Zealand: *Nothofagus fusca*, *N. truncata* and *N. solandri*. Only one variety of *N. solandri* was consistently collected to avoid any potential confounding effects caused by variation within the species. *N. solandri* var. *cliffortioides* was preferred to *N. solandri* var. *solandri* due to the former's more extensive distribution in the South Island. In the southern half of the island *N. solandri* var. *solandri* is absent, although intermediate forms between it and *N. solandri* var. *cliffortioides* are widely scattered (Wardle 1984).

6.1 Sampling

Sampling sites were chosen to (i) include the widest practicable geographical spread across the South Island and (ii) include at least 3-4 samples of each species on either side of the disjunction. Additionally an outgroup for each species was sampled from North Island provenances. Given that to the south of the disjunction, *N. truncata* is known only from a few nearby localities, only two samples (from the same population) were tested. The criterion of widest possible geographic spread for *N. solandri* var. *cliffortioides* was compromised to an extent on the south side of the disjunction. With the long-distance pollen dispersal and hybridisation hypothesis (Section 3.5) in mind, samples of *N. solandri* var. *cliffortioides* were limited to the same valley systems as those of *N. fusca*. This is based on the assumption that individuals of the reconstituted *N. fusca* could still be in close proximity to the *N. solandri* var. *cliffortioides* populations from which their maternal ancestry was derived.

Locations of sampling sites are shown in Fig. 6.1. Details of sample sites are in Tables 6.1, 6.2 and 6.3.

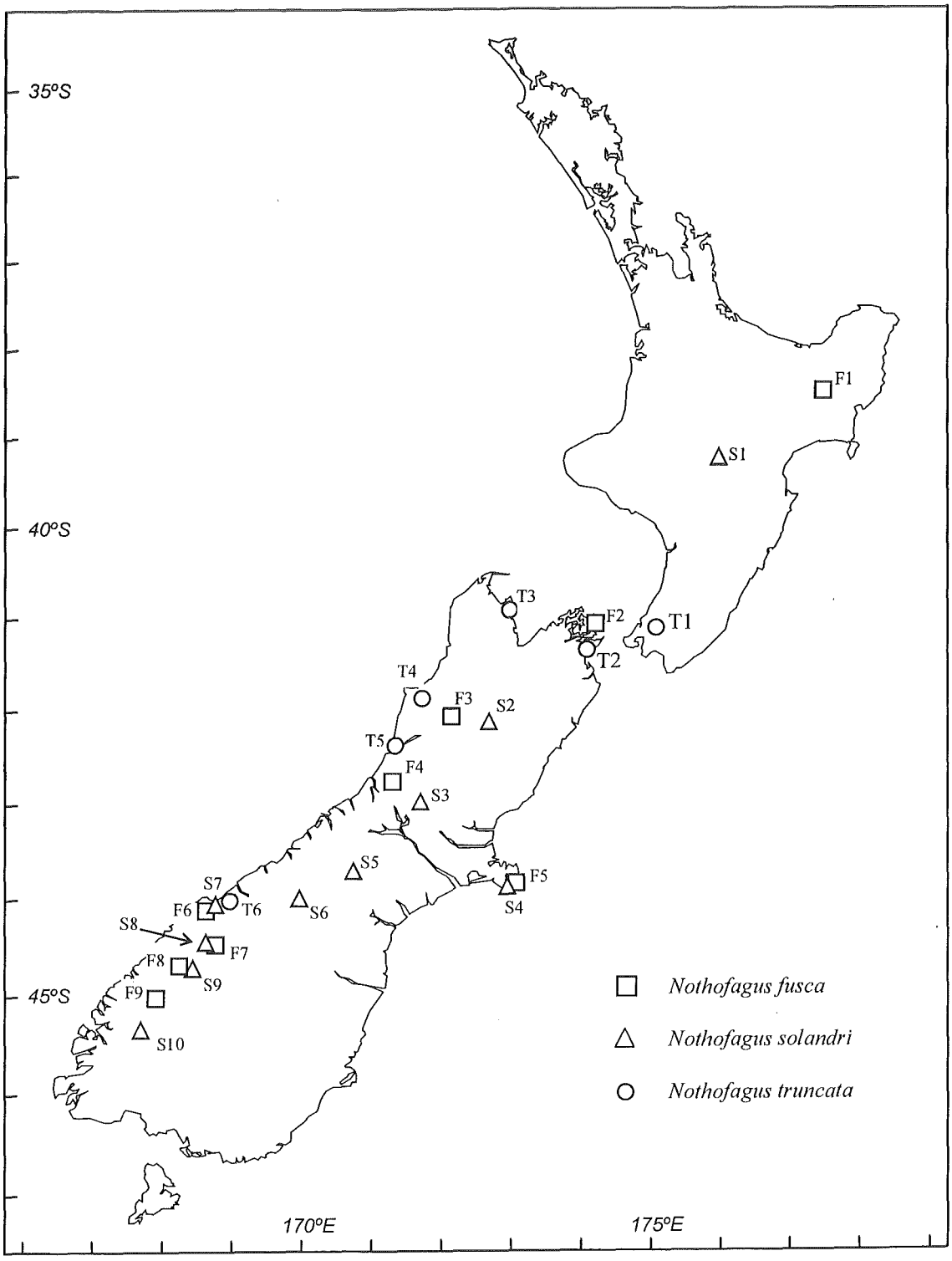

Fig 6.1 Sampling sites for *Nothofagus fusca*, *N. solandri* var. *cliffortioides* and *N. truncata*. Refer to Tables 6.1, 6.2 and 6.3.

Table 6.1 Collection sites for *Nothofagus fusca*

Site code	Site Name	Region	Map / Grid Ref	Latitude	Longitude	Altitude (metres)	Habitat	Collector / date collected
F1	Moanui *	Upper Waioeka	X17 950005	S 38° 25'	E 177° 25'	600		T. Thomsen 17/09/01
F2	Mt Stokes	Marlborough Sounds	P26 040127	S 41° 05' 13"	E 174° 07' 08"	750	*N. fusca* / *N. menziesii* forest	T. Thomsen 10/01/01
F3	Station Creek	Maruia Valley	L31 492892	S 42° 12' 07"	E 172° 15' 50"	400	*N. fusca* / *N. menziesii* forest	T. Thomsen 18/11/00
F4	Deception River footbridge	Otira Valley	K33 959233	S 42° 47' 21"	E 171° 36' 19"	300	Mixed *N. fusca* / podocarp forest	T. Thomsen 03/01/01
F5	West Track, Hinewai Reserve	Akaroa	N36 112106	S 43° 48' 43"	E 173° 00' 53"	520	Mixed *N. fusca* / hardwood forest	T. Thomsen 23/12/01
F6	Jackson River	Jackson Bay	E38 578682	S 44° 06' 35"	E 168° 35' 54"	80	Podocarp / hardwood forest	T. Thomsen 21/12/00
F7	Glacier Burn	East Matukituki	F39 761310	S 44° 27' 09"	E 168° 48' 11"	380	*N. fusca* forest	T. Thomsen 20/12/00
F8	Paradise	Dart Valley	E40 417018	S 44° 41' 54"	E 168° 21' 03"	390	*N. fusca* forest	T. Thomsen 18/12/00
F9	Lake Gunn	Upper Eglinton	D41 230828	S 44° 51' 33"	E 168° 06' 04"	480	*N. fusca* / *N. menziesii* forest	T. Thomsen 17/12/00

* Collected from former Forest Research Institute site, Rangiora

Table 6.2 Collection sites for *Nothofagus solandri* var. *cliffortioides*

Site code	Site Name	Region	Map / Grid Ref.	Latitude	Longitude	Altitude (metres)	Habitat	Collector / date collected
S1	Desert Road *	Tongariro River	T19 490230	S 39° 09' 51"	E 175° 45' 56"	760		T. Thomsen 17/09/01
S2	Princess Stream	Lake Tennyson	M31 863885	S 42° 12' 37"	E 172° 42' 47"	1240	*N. solandri* var. *cliffortioides* forest	T. Thomsen 03/02/02
S3	Broken River skifield road *	Craigieburns	K34 038850	S 43° 08' 06"	E 171° 41' 40"	1000		T. Thomsen 11/12/01
S4	Armstrong Reserve	Akaroa	N36 097081	S 43° 50' 04"	E 172° 59' 46"	620	*N. solandri* var. *cliffortioides* forest	T. Thomsen 23/12/01
S5	Scour Stream	Mesopotamia, Rangitata	J36 375246	S 43° 39' 58"	E 170° 51' 39"	750	*N. solandri* var. *cliffortioides* forest	T. Thomsen 16/02/02
S6	Bush Stream	Mount Cook	H37 767011	S 43° 51' 40"	E 170° 05' 50"	660	*N. solandri* var. *cliffortioides* forest	T. Thomsen 17/02/02
S7	Jackson River	Jackson Bay	E38 655742	S 44° 03' 34"	E 168° 41' 54"	10	Mixed *N. solandri* var. *cliffortioides* / podocarp forest	T. Thomsen 21/12/00
S8	Cascade Saddle Track	West Matukituki	E39 646267	S 44° 29' 09"	E 168° 39' 21"	750	*N. solandri* var. *cliffortioides* forest	T. Thomsen 20/12/00
S9	Muddy Spur	Rees Valley	E40 523004	S 44° 42' 58"	E 168° 29' 00"	1100	*N. solandri* var. *cliffortioides* forest	T. Thomsen 19/12/00
S10	Ten Mile Bush	Te Anau	D42 015323	S 45° 18' 03"	E 167° 47' 20"	210	*N. solandri* var. *cliffortioides* forest	T. Thomsen 18/12/00

* Collected from former Forest Research Institute site, Rangiora

Table 6.3 Collection sites for *Nothofagus truncata*

Site code	Site Name	Region	Map / Grid Ref.	Latitude	Longitude	Altitude (metres)	Habitat	Collector / date collected
T1	Te Marua lookout	Hutt Valley	S26 905110	S 41° 05' 16"	E 175° 08' 55"	200	Mixed *Nothofagus* / scrub	S. Trewick 03/01/02
T2	Shakespeare Bay	Picton	P27 933925	S 41° 16' 12"	E 173° 59' 39"	100	Mixed *Nothofagus* / broadleaf forest	T. Thomsen 01/03/02
T3	Totaranui	Abel Tasman National Park	N25 087409	S 40° 50' 18"	E 172° 59' 05"	200	Mixed *Nothofagus* / broadleaf forest	S. Trewick 01/03/02
T4	Hawks Crag	Lower Buller Gorge	K29 085263	S 41° 51' 49"	E 171° 46' 39"	30	Mixed *Nothofagus* / broadleaf forest	T. Thomsen 02/03/02
T5	Taylorville	Lower Grey River	J31 699607	S 42° 26' 53"	E 171° 17' 48"	20	Mixed *Nothofagus* / broadleaf forest	T. Thomsen 03/03/02
T6	Nisson Hill **	Jackson Bay	F37 735805	S 44° 00' 23"	E 168° 48' 08"	60		P. Garnock-Jones 04/03/02

** Collected from transplanted seedlings in Wellington

As far as possible sites with a single *Fuscospora* species were chosen, so that the possibility of hybridisation was minimised. Such sites were identified with varying ease, and were most easily found where habitats strongly favoured one or other of the species. This was readily achieved for *N. solandri* var. *cliffortioides* which could often be collected in environments well beyond the tolerances of *N. fusca* and *N. truncata*. The best sites for this species tended to be drier environments above 800 metres, where it often formed pure stands distant from the other *Fuscospora* species. The best sites for *N. fusca* tended to be fertile and moist valley floor or lower slopes, where it often formed pure stands or co-dominated with *N. menziesii*. It was more difficult to find such sites for *N. truncata*. This species was usually found on low spurs, sometimes adjacent to rivers, although *N. fusca* was often in the vicinity.

The taxonomic integrity of samples was verified by comparison of their leaf morphology with drawings (Fig. 6.2) and descriptions in Wardle (1984).

N. truncata specimens were collected from the roadside. All other specimens were collected by permission of the landowner or agent. All specimens on Crown land were collected by permission of the Department of Conservation, Permit Number 15 15/12/2000. The *N. fusca* sample at Station Creek was collected by permission of Timberlands West Coast Ltd., Access Permit number 17730. Five samples were not collected *in situ*, but from specimens in cultivation known to be sourced from the described area. These included:

(i) Three *N. fusca* and *N. solandri* var. *cliffortioides* samples collected from the former Forest Research Institute site at Oxford Road, 1 kilometre to the west of Rangiora. These had been collected as seeds from different localities around New Zealand in autumn 1979, and germinated and planted out in experimental plots in January 1980 (Wilcox and Ledgard 1983). Specimens were positively identified by a plastic label denoting place of origin, and cross-referenced to the study of Wilcox and Ledgard (1983) from which latitude and longitude were obtained.

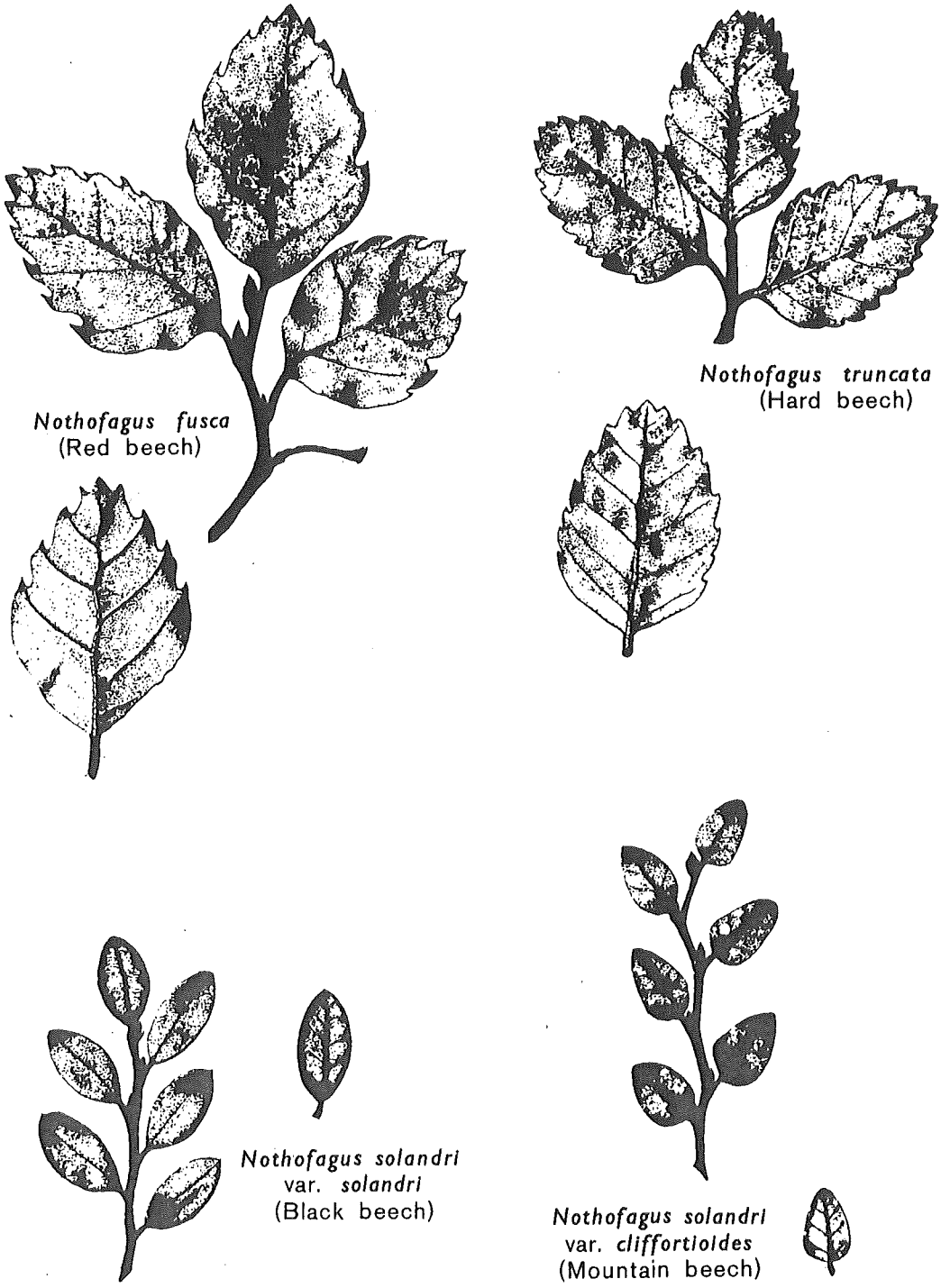

Fig. 6.2 Leaves of the New Zealand *Nothofagus* subgenus *Fuscospora* species. Reproduced from Wardle (1984)

(ii) Two South Westland samples of *N. truncata*, removed as seedlings by P. Garnock-Jones from Nisson Hill in 1993 (specimen held at the Landcare Herbarium, Landcare Research, Lincoln CHR 470268), and since grown in containers which are now in Wellington. These were provided by P. Garnock-Jones.

Where possible, new leaves and leaf shoots were collected from the lower branches of adult trees. New growth, with lower accumulations of secondary compounds, is more likely to yield a higher volume of quality DNA (Dessauer et al. 1996; S. Wagstaff pers. comm.). Where new growth was not available, leaves were collected from the tips of branchlets.

Five to ten leaves were collected per tree, and stored in small airtight plastic bags with 5-10 gm of silica gel. During the collection phase, silica gel was inspected daily, and on translucence or pinking, was replaced. On return to the University of Canterbury, silica gel was removed from samples, which were then stored at -20 degrees C. Voucher specimens consisting of small branchlets were taken and stored in moistened plastic bags, and transferred to a press upon return to the University of Canterbury. Vouchers have been placed in the herbarium at the University of Canterbury (CANU).

Location of collection sites were recorded on an Infomap 260 series, with coordinates to the nearest 0.1 km. Coordinates were converted to latitude and longitude (Table 6.1) using GARtrip Version 202 (SR-2) on a Windows platform (Tables 6.1, 6.2 and 6.3).

6.2 DNA extraction and quantitation

Total DNA was extracted from leaves using the CTAB technique. This technique has been widely used to successfully obtain total DNA from a range of plant species. CTAB is a cationic detergent which dissolves membranes and forms a complex with DNA (Weising et al. 1995).

The 2x CTAB extraction buffer recipe:

0.2M Tris-HCl

1.4M NaCl

0.5M EDTA (ethylenediaminetetraacetic acid)-disodium salt

2% CTAB (w/v) (hexadecyltrimethylammonium bromide)

1% PVP-40 (w/v)

Just before use, 0.2% (v/v) β-mercaptoethanol was added.

Extraction consisted of the following steps:

1) Cellular breakdown: An approximately 1 cm^2 segment of leaf, with liquid nitrogen added, was ground with unheated mortar and pestle until powdery. 1 ml of CTAB extraction buffer was added, and grinding continued until lysed cellular matter was in solution. Around 750 µl of the solution was incubated in a 1.5 ml microtube on a heat block at 65 deg C for 30 to 60 minutes.

2) Separation of nucleic acids. A solution of chloroform and amylalcohol (mixed in ratio (v/v) of 24:1) was added in ratio (v/v) of 1:1 with the solution, and gently mixed. Proteins and lipids were separated out with the chloroform amylalcohol solution by centrifuging at 8000 rpm for 4 minutes. The extract buffer solution was aliquoted and the separation process was repeated.

3) Precipitation of nucleic acids: Isoproponol and the solution were gently mixed in ratio (v/v) of 2:3, and stored at -20° C overnight. Centrifuging at 13,000 rpm for 10 minutes at 4° C separated out nucleic acids into a pellet. Supernatants were drained out, and the pellet was washed with 300 µl 70% ethanol. Following

centrifuging at 13,000 rpm for 2 minutes, supernatants were drained out and the pellet was left to air dry (occasionally on heat block).

4) Resuspension. The pellet was resuspended in 20 µl TE buffer (10mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0) for one hour on a heat block at 65° C.

Quantitation was carried out by running on a gel with comparison to varying concentrations of Lambda DNA.

6.3 Amplification of cpDNA regions using PCR

The amplification of homologous regions of DNA relies on the use of specific primers, oligonucleotides of around 20 base pairs, that are known to anneal to single known locations on the genome. Pairs of specific primers are used together, and are designed to delimit the region of DNA to be amplified, by annealing close to one another, but on different strands (Palumbi 1996). The two primers thus initiate the copying of the DNA strand lying between them, in this case a section of more rapidly evolving, noncoding cpDNA (Section 4.3). Amplified sections are subsequently the basis for sequencing (Section 6.4) and RFLP assays (Section 6.5).

6.3.1 Selection of cpDNA primer pairs

At the start of this study, there was no record of primers successfully amplifying non-coding cpDNA regions in *Fuscospora* species. The selection of primer pairs for testing was therefore based on two main criteria:

1. The likelihood of their successfully producing amplified cpDNA.

   This was best achieved by selecting universal primers, which are known to work for a number of other taxa, particularly those closely related to *Nothofagus*. Universal primers have been designed (Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapègue et al. 1997a) through the alignment of the complete sequences of the chloroplast genome of three disparate land plants: the rice *Oryza sativa* (a monocot), the tobacco *Nicotiana tabacum* (a dicot) and the liverwort *Marchantia*

polymorpha (a bryophyte). Many of the universal primer pairs have successfully amplified target regions of cpDNA across a very broad range of plant taxa including angiosperms, gymnosperms, pteridophyes and bryophytes, as well as algae (Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapègue et al. 1997a).

Further confidence could be placed in universal primer pairs if they had been successfully used to amplify cpDNA regions in taxa closely related to *Fuscospora*. This has often been the case in Fagaceae (eg Demesure et al. 1995; Dumolin-Lapègue et al. 1997b) and in a single *Nothofagus* study (Marchelli et al. 1998).

The greater the distance (in base pairs) between the two primers of a pair (ie the longer the cpDNA region to be amplified), the greater the difficulty in making successful amplications work (S. Wagstaff pers. comm.). Consequently primer pairs expected to span shorter regions were favoured. These concerns were borne out, since several longer cpDNA regions could not be amplified in this project.

2. The likelihood that amplified DNA would reveal infraspecific polymorphisms.

To determine the most likely candidate cpDNA regions for revealing polymorphisms, universal cpDNA primers (Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapègue et al. 1997a) were compared from a range of infraspecific plant studies from the literature. There was considerable variability in the success rate of different cpDNA regions; RFLP repeatedly revealed polymorphisms in some regions, whilst consistently failing to show polymorphisms in others. Extra weight was given to those cpDNA regions where polymorphisms were revealed for taxa closely related to *Fuscospora* (eg other *Nothofagus*, Fagaceae).

Although longer cpDNA regions tended to most commonly reveal RFLP polymorphisms, this was not always the case. This may be because:

- Rates of evolution are not constant across all non-coding regions, and thus variation is not distributed evenly between these regions.
- The various regions amplified by universal primers have varying degrees of conservative genetic code embedded.

- The region may have a low number of recognition sites for restriction endonucleases.

In general, long cpDNA regions tend to be advantageous from the RFLP standpoint, since they tend to have more recognition sites. Long regions do not, however, offer advantages for sequencing, because sequences of more than around 1000 bp will require the use of extra sequencing reactions using internal primers. In terms of utility for RFLP, there is a trade-off between the ease of amplifying shorter regions versus the greater probability of longer regions yielding variation.

Consequently, several primers were designed specifically for this project to amplify subsets of longer cpDNA regions. Each of these was paired with a universal primer to amplify a short section at one end of the region. The specially designed primers were based on the cpDNA sequence for *Lotus japonicus* (Fabaceae) (Genbank accession no. AP002983). Of all of the taxa with a fully sequenced chloroplast genome on Genbank, this is suggested by recent phylogenetic analyses, based on cladistic methods and molecular sequence data, to be the closest relative to *Nothofagus* (The Angiosperm Phylogeny Group 1998). Designed primers were (as for universal primers) designed to bind to genetic coding areas since these are the most conserved. They were oriented to pair with a universal primer to span a short non-coding region of chloroplast DNA. The primers perfectly matched homologous sequences in *Nicotiana tabacum* (Solanaceae) (Genbank accession no., NC_001879), confirming their highly conserved nature and their probable suitability for amplification of *Nothofagus* cpDNA.

Eleven primers were tested (Table 6.4) in seven combinations of pairs (Table 6.5).

Table 6.4 Primers used to amplify regions of non-coding cpDNA

Number	Gene	Sequence 5' - 3'	Designer
1	*trn*T	CATTACAAATGCGATGCTCT	Taberlet et al. (1991)
2	*trn*L	CGAAATCGGTAGACGCTACG	Taberlet et al. (1991)
3	*trn*F	ATTTGAACTGGTGACACGAG	Taberlet et al., (1991)
4	*trn*D	ACCAATTGAACTACAATCCC	Demesure et al. (1995)
5	*trn*T	CTACCACTGAGTTAAAAGGG	Demesure et al. (1995)
6	*trn*E	AACCGCTAGACGATGGGGGC	T. Thomsen
7	*trn*F	CTCGTGTCACCAGTTCAAAT	Dumolin-Lapègue et al. (1997)
8	*trn*V	CCGAGAAGGTCTACGGTTCG	Dumolin-Lapègue et al. (1997)
9	*ndh*J	TTATGAAATACAAGATGCTC	T. Thomsen
10	*trn*K	GGGGTTGCTAACTCAACGG	Johnson and Soltis (1994)
11	*mat*K	TATTCTGTTGATACATTCG	R. Bayer (Chandler et al. 2001)

Table 6.5 Primer pairs tested for amplification of regions of non-coding cpDNA. Numbers in parentheses refer to primer numbers in Table 6.4.

Primer 1	Primer 2
*trn*T (1)	*trn*F (3)
*trn*L (2)	*trn*F (3)
*trn*D (4)	*trn*T (5)
*trn*E (6)	*trn*T (5)
*trn*F (7)	*trn*V (8)
*trn*F (7)	*ndh*J (9)
*trn*K (10)	*mat*K (11)

6.3.2 PCR amplification

Amplification procedures using the Polymerase Chain Reaction (PCR) were subject to experimentation in order to optimise amplification success rates, and in an attempt to consistently produce high quality amplified DNA product. A range of reagent proportions produced acceptable results; the following mix was reliable and was utilised in this project:

For a 25 µl reaction (constituents added in the order given):

Constituent	Quantity (µl)
Distilled water (dH_2O)	15.3
PCR reaction buffer 10x conc	2.5
$MgCl_2$ Stock Solution (25mM/l)	2.5
PCR Nucleotide Mix (dNTP), 10mM each dNTP	2.0
Primer #1 (10 µM)	1.0
Primer #2 (10 µM)	1.0
Taq DNA Polymerase, 1 Unit	0.2
Template DNA	0.5

Reactions for a given primer pair were always performed in batches. For each batch, a cocktail of all reagents except template DNA was initially mixed in a 1.5 ml microtube. Reagent quantities per cocktail were the above quantities multiplied by the number of reactions in the batch. Each PCR tube received 24.5 µl of cocktail, followed by the template DNA. Reactions always had a negative control (no template DNA). A positive control was also often included to assess cocktail quality, or when template DNA was suspect in failed reactions.

Thermal cycling reactions were performed on an MJ Research PTC-200 Peltier Thermal Cycler. Thermal cycles conformed to standard denaturing, annealing and extension phases. However two settings varied between primer pairs, having to be fine-tuned according to the characteristics of the oligonucleotide primer pairs, and to the length of the cpDNA region being amplified:

(i) Annealing temperature were set according to the length and the nucleotide composition of the oligonucleotides. It is critical in producing workable DNA product that the annealing temperature be set low enough so that a high number of oligonucleotides bind to the designated primer sites, and to these sites only (Palumbi 1996). However, as annealing temperature decreases further, specificity of primer binding also decreases, and oligonucleotides may additionally bind to poorer-matching, non-target sites. This may therefore result in the synthesis of alternative PCR products (Palumbi 1996). One rule of thumb is for annealing temperature to be equal to the approximate calculation for T_m, the temperature (deg C) at which half of the potential binding sites (with perfect matches) should have primer bound to them. T_m = (4 * no. of G's and C's in the primer sequence) + (2 * no. of A's and T's in the primer sequence). In practice, successful annealing temperatures in this project tended to be lower than estimates derived for T_m. This may be a consequence of using universal primers, since they will seldom provide a perfect match with target sequences (Palumbi 1996).

(ii) The need for extension time to be long enough to allow complete polymerisation of nucleotide bases to daughter DNA strands (Palumbi 1996). The longer the strand, the longer the time needed for Taq polymerase to perform a complete synthesis. Palumbi (1996) suggests 30 second extensions for products under 500 bp; 60 seconds for 500-1500 bp products, and 90 seconds for longer products.

As an example, the thermal cycle to amplify *trn*L-*trn*F (~950bp) was:

STAGE	TEMPERATURE (°C)	DURATION (min:sec)
At start		
Denature:	94	4:00
Cycle (30 times)		
Denature	94	1:00
Anneal	48	1:00
Extension	72	2:00
At end		
Extension	72	10:00

PCR amplification was tested using gel electrophoresis. 4 µl per sample was run out on a 1% agarose gel at 80V for 40-50 minutes. The standard was 5 µl of Gibco BRL 1 Kb Plus DNA Ladder (1 µg/µl), in ratio (v/v) of 3:9:88 with loading buffer and TE respectively. The gel was stained in 0.5 µg/ml working concentration of Ethidium Bromide.

A correct PCR amplification was demonstrated by a single band, indicating equal-length fragments consistent with amplification of a single DNA region. The length could be verified to some extent through comparison with the cpDNA region in question from other taxa (either from other studies or from Genbank). However, this check was only approximate, since the length of intergenic spacers varies between taxa.

6.4 Sequencing of cpDNA regions

The sequencing method used is derived from Sanger et al. (1977), but with modifications to permit greater efficiency and automatic sequencing. The technique elongates DNA molecules using DNA polymerase, as in normal PCR amplification, although the end result is the generation of a series of DNA molecules of the same

sequence but of different lengths (Klug and Cummings 1994; Hillis et al. 1996). The DNA strand to be sequenced is denatured, and single strands are used as templates for the synthesis of a new DNA strands. As in PCR amplification, a primer anneals to a specific site on the template, and DNA polymerase adds deoxynucleotide dNTPs to the growing strand. Along with dNTPs, a smaller number of dideoxynucleotides (ddNTPs), corresponding to the four types of deoxynucleotides, are used as chain-terminators (Hillis et al. 1996). As for dNTPs, ddNTPs can be added to a growing strand, but because they lack a 3' OH group, further nucleotides can't be added to them and a termination of strand synthesis occurs (Hillis et al. 1996). Thus the newly synthesised strands are randomly terminated, resulting in a collection of differently sized fragments (Hillis et al. 1996), which end at As, Gs, Cs and Ts respectively. To yield a population of fragments terminating at all potential nucleotide sites within a few hundred bases from the primer requires a ratio of dNTPs to ddNTPs of around 100:1 (Snustad and Simmons 2000).

In automatic sequencing, different fluorescent dyes are used to label each of the four different ddNTPs (Snustad and Simmons 2000). This distinguishes the DNA fragments of different length after they are separated out on high-resolution polyacylamide gels, allowing them to be detectable by photocells (Snustad and Simmons 2000). The detected sequence is recorded directly to a chromatograph.

A single sequence reaction normally provides 400-500 bp of quality sequence. All cpDNA regions sequenced in this project are in the range of 600-950 bp, and therefore full sequences of a region required at least two sequencing reactions. These were normally sequenced from the primers at each end (ie delineating the cpDNA region). The complementary chromatograms between them usually provided enough information to resolve any ambiguities arising from inferior signals. The first part of a sequence (at the primer end) often had an unreadable signal; in order, therefore, to sequence the extremities of a cpDNA region it was necessary to run sequencing reactions from primers internal to the region.

6.4.1 Purification of amplification product

Purification was carried out using the High Pure PCR Product Purification Kit (Roche), with some modifications to the manufacturer's recommended procedures.

Binding buffer was added in ratio (v/v) of approximately 5:1 to amplification product (normally 100 µl Binding Buffer to 20 µl of amplification product), and these were centrifuged in a polypropylene filter tube at 14,000 rpm for 60 seconds. For the wash step, 500 µl of Wash Buffer was added and centrifuged at 14,000 rpm for 60 seconds; this step was repeated with 200 µl of Wash Buffer. Elution was carried out with 25 µl warm distilled water, which was left to sit for 60 seconds before centrifuging at 14,000 rpm for 60 seconds.

6.4.2 Sequencing reactions

Sequencing of a region from a given primer was performed from a single reaction. Each of the four different dideoxynucleotides (ddNTPs) was tagged by a different dye label, allowing analysis on a single gel lane in the automatic sequencing process.

The 'terminator mix' consisted of deoxynucleotides (dNTPs), fluorescent dideoxynucleotides (ddNTPs) and Taq Polymerase. Two terminator mixes were utilised during this project: ABI PRISM® Big Dye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (including all sequencing carried out at Landcare Research) and DYEnamic™ ET Terminator (Amersham Pharmacia Biotech).

For a 10 µl reaction (added in the order given):

Constituent	Quantity (µl)
'Terminator mix' (Big Dye v3.0 or DYEnamic)	2
10 µM primer	1
PCR product	2 - 7
Distilled water (dH_2O)	0 - 5

The quantity of PCR product (and therefore of dH_2O) was adjusted according to its quality as visualised by electrophoresis following amplification (Section 6.3.2).

The thermal cycle used to perform the sequencing reaction was:

STAGE	TEMPERATURE (°C)	DURATION (min:sec)
Cycle (30 times)		
Denature	95	0:10
Anneal	50	0:10
Extension	60	2:00

6.4.3 Purification of sequencing reaction product

It is necessary to remove dye-tagged terminators from the samples prior to electrophoresis, since these can interfere with signals from the shorter DNA fragments and therefore obscure data. Precipitation methods were used since they are cheap and fast, although they may remove fewer dye-tagged terminators. The method used differed depending on whether the terminator mix in the sequencing reaction was BigDye v3.0 or DYEnamic.

For sequencing reactions using BigDye v3.0, 1.0 µl 3M Sodium Acetate pH 4.8 and 25 µl 95% ethanol were added to the reaction mixture. This was mixed and sat on ice for 10 minutes before centrifuging at 13,000 rpm for 30 minutes. Supernatants were aspirated and 125 µl 70% ethanol was added prior to centrifuging at 13,000 rpm for 10 minutes. Supernatants were aspirated and the tube was left to air dry.

For sequencing reactions using DYEnamic, 1.0 µl 7.5M Ammonium Acetate and 28 µl 100% ethanol were added to the reaction mixture. This was vortexed, and the mixture was stored at 4° C for 30 minutes before centrifuging at 13,000 rpm for 20 minutes. Supernatants were aspirated and 100 µl 70% ethanol was added prior to centrifuging at 13,000 rpm for 10 minutes. Supernatants were aspirated and the tube was left to air dry.

6.4.4 Automatic sequencing

Electrophoresis and automatic sequencing were carried out by the Waikato DNA Sequencing Facility at the School of Biological Sciences, The University of Waikato, Hamilton.

6.4.5 Editing of interpreted sequence data

Chromatograms and interpreted DNA sequences were retrieved from the Waikato DNA Sequencing Facility FTP folder. Interpreted sequences needed to be edited, because of ambiguities arising from the chromatogram. Ambiguities arose as a result of an inability to distinguish a weak signal from background noise (Hillis et al. 1996), poorly-defined peaks (Hillis et al. 1996) and superimposed dye peaks (S. Wagstaff pers. comm.).

For sequences processed at Landcare Research, editing of interpreted DNA sequences was performed using Sequencher^{TM} version 3.0 (1995 Gene Codes Corporation) on a Macintosh platform. For sequences processed at the University of Canterbury, editing was performed using Seq Ed^{TM} version 1.0.3 (1992 Applied Biosystems Inc.) on a Macintosh platform.

Sequences were compared using the program Clustal X on a Microsoft Windows NT platform. This allowed the alignment of multiple sequences with colour-coding by base type to allow quick comparison between sequences.

6.5 RFLP assays of cpDNA regions

6.5.1 Selection of restriction enzymes

The cutting of PCR amplification products by restriction enzymes is a fast and economical way of assaying for polymorphisms across a number of cpDNA regions for a large number of individuals (Section 4.5). However, a given enzyme can only sample a small proportion (if any) of the amplified region, those sites where its recognition sequence falls. Consequently a large number of enzymes can be fruitlessly assayed. To bypass this, program Sequencher v. 3.0 was used to map all cutting sites for 71 commonly available restriction enzymes across sequenced cpDNA regions. This was done for individuals from two species, north and south of the disjunction:

- F3 (*Nothofagus fusca*, Maruia Valley; north of disjunction),
- F9 (*N. fusca*, Upper Eglinton valley; south of disjunction)
- S9 (*N. solandri* var. *cliffortioides*, Rees Valley; south of disjunction)
- S10 (*N. solandri* var. *cliffortioides*, Te Anau; south of disjunction)

The cutting site information indicated immediately the utility of each enzyme for the DNA region, by indicating the number of fragments, and the length of each fragment. More importantly, however, it also indicated whether recognition sequences and mutations (substitutions or indels) coincide, and therefore whether the enzyme would produce fragment length polymorphisms.

6.5.2 Purification of amplification product

Prior to the restriction digest, PCR amplification product was purified using Bio-Rad Prep-A-Gene® DNA Purification Systems. 30 µl PCR product, 105 µl binding buffer and 5 µl silica matrix were mixed and incubated at room temperature for 10 minutes. Following centrifuging at 13,000 rpm for 10 minutes, the supernatant was aspirated, and the pellet rinsed with 125 µl binding buffer. After centrifuging at 13,000 rpm for

30 seconds, the supernatant was aspirated and the pellet was washed with 125 µl wash buffer. After centrifuging at 13,000 rpm for 30 seconds, the supernatant was aspirated and the wash step was repeated. The bound DNA was eluted by adding 15 µl TE, and incubating at 37° C for 10 minutes, before removing the supernatant.

6.5.3 Restriction digests

For a 10 µl digest (added in the order given):

Constituent	Quantity (µl)
Distilled water (dH_2O)	6.8
Buffer 10x conc.	1.0
Enzyme (5 units)	0.2
Purified amplified DNA	2.0

Digests were always performed in batches. For each batch, a cocktail of all reagents except amplified DNA was initially mixed in a 1.5 ml microtube. Reagent quantities per cocktail were equal to the quantities above multiplied by the number of reactions in the batch. Each PCR tube received 8 µl of cocktail, followed by the amplified DNA. Digests were run in an incubator at 37° C for 3 - 4 hours, or overnight.

Digestions were tested using gel electrophoresis. The 10 µl sample was run out on a 2.5% 3:1 agarose gel at 70V for 60 minutes. The standard was 5 µl of Gibco BRL 1 Kb Plus DNA Ladder (1 µg/µl), in ratio (v/v) of 3:9:88 with loading buffer and TE respectively. The gel was stained in 0.5 µg/ml working concentration of Ethidium Bromide.

For verification, the fragment sizes were compared to the expected fragment sizes predicted by program Sequencher v. 3.0 (Section 6.5.1).

7 Results

7.1 DNA extractions and quantitation

Quantitative tests, with comparisons to varying concentrations of Lambda DNA (Section 6.2), indicated a high variation in concentration of DNA extracted via the CTAB technique. The highest concentrations were in the order of 0.25 μg/μl. Some extracted DNA could not be visualised at all on agarose gels, and yet the template was still adequate to produce amplified cpDNA via PCR. For these samples, concentrations were inferred to be <0.025 μg/μl.

7.2 Amplification of cpDNA regions

Successful amplification (eg Fig. 7.1) occurred for four of the seven primer pairs tested (Table 7.1). No amplification occurred for two primer pairs, *trn*T - *trn*F and *trn*F - *trn*V, which may be because of the long length of these regions. In other studies, *trn*T - *trn*F amplified a 1,900 bp region for *Fagus* (Petit et al. 1997), and *trn*F - *trn*V a 3,000 bp region for *Nothofagus* (Marchelli et al. 1998). Another primer pair, *trn*D - *trn*T, amplified two products of length 1300 bp and 300 bp (Fig. 7.2). The longer of the two products is assumed to be the target region, since amplifications of the same region yielded 1800 bp in *Fagus* (Petit et al. 1997) and 1100 bp in *Nothofagus* (Marchelli et al. 1998). The shorter product may be due to non-target annealing (Section 6.3.2). Attempts to eliminate this by raising the annealing temperature were unsuccessful, since at no temperature was the longer product present and the shorter product absent.

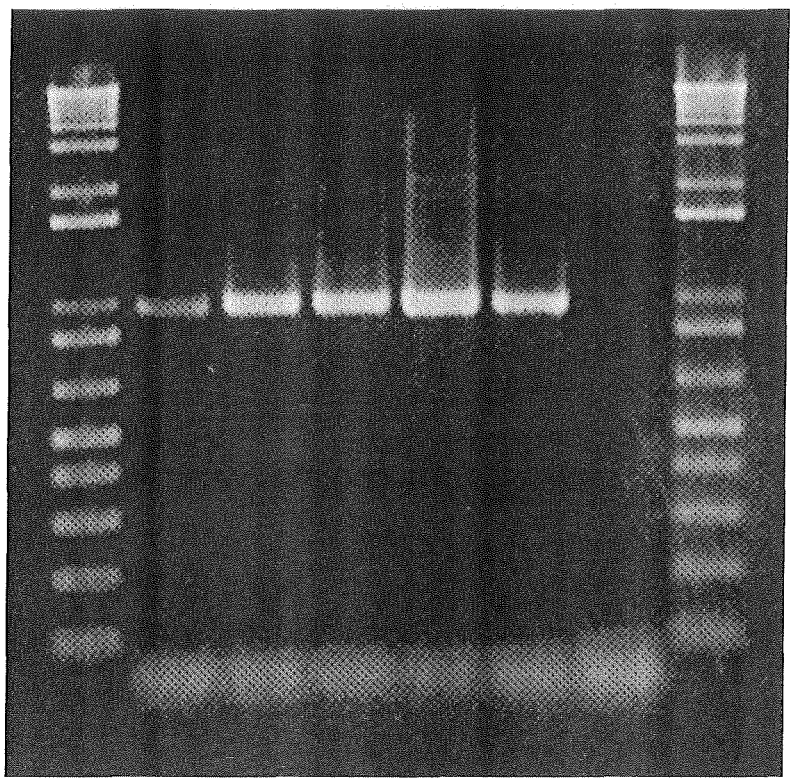

Fig. 7.1 PCR amplification of the *trn*L-*trn*F cpDNA region, as visualised on a 1% agarose gel. Lanes 1 and 8 are 1Kb Plus Ladder. Lane 2 is *Nothofagus fusca*, Kaweka Range (not subsequently used). Lanes 3-6 are S8, S7, S3 and S1. Lane 7 is negative control.

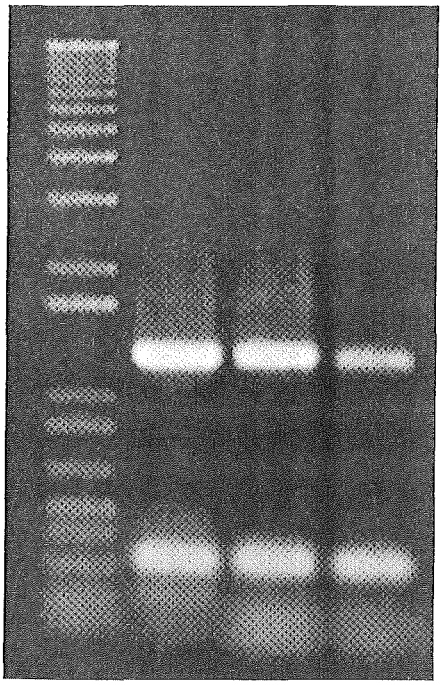

Fig 7.2 PCR amplification of the *trn*D-*trn*T region, visualised in a 1% agarose gel. Lane 1 is 1 Kb Plus ladder. Lanes 2 and 3 are F9 (different template concentrations). Lane 4 is S10. Bands are 1300 and 300 base pairs.

Table 7.1 Results of cpDNA amplification tests for all regions assayed. Primer numbers in parentheses refer to primer numbers in Table 6.4. Annealing temperature and extension time is presented for successful assays only. Length is determined from sequencing, and is amplified length, excluding primers.

Primer 1	Primer 2	Amplification Product (Y/N)	Annealing temp (°C)	Extension time (min:sec)	Length (base pairs)
*trn*T (1)	*trn*F (3)	N			
*trn*L (2)	*trn*F (3)	Y	48	2:00	943
*trn*D (4)	*trn*T (5)	Y	52	2:30	1300, 300
*trn*E (6)	*trn*T (5)	Y	48	2:00	522
*trn*F (7)	*trn*V (8)	N			
*trn*F (7)	*ndh*J (9)	Y	48	2:00	660
*trn*K (10)	*mat*K (11)	Y	48	2:00	951

7.3 Sequencing of cpDNA regions

Initially, each of the four successfully amplified cpDNA regions was fully, or almost fully, sequenced This was done for three or four individuals from two species across a wide geographic range (Section 6.5.1). Only one cpDNA region (*trn*L - *trn*F) yielded a polymorphism. Further sequencing was concentrated on this cpDNA region only, to establish a geographical distribution for this polymorphism.

Details of sequencing reactions for each cpDNA region follow:

1. *trn*L - *trn*F

Initial sequencing reactions, utilising two internal and two external primers (Fig. 7.3), were used to produce sequences across all of this cpDNA region for F3, F9, S9 and S10 (Appendix 2). The two external primers, 'c' and 'f' are the amplification primers (2) and (3) respectively (Table 6.4). The sequences (5' - 3') for internal primers 'd' and 'e' are GGGGATAGAGGGACTTGAAC and GGTTCAAGTCCCTCTATCCC respectively.

Sequencing revealed a single polymorphism in the region. To maximise the number of samples that could be tested, later sequencing tended to utilise only the two external primers ('c' and 'f'), which produced sequences across the entire region except at the extremes, near the primers. After sampling across a wide geographical range revealed no further polymorphisms, sequencing of further samples was concentrated on determining the geographical distribution of the single polymorphism found. Consequently a single internal primer ('e'), which consistently produced good quality sequence data at the polymorphic section of DNA, was used. In total 25 samples were sequenced to a varying extent across this cpDNA region (Table 7.2).

The single polymorphism was within a mononucleotide repeating sequence of Ts in positions 838-844, within the *trn*L-*trn*F intergenic spacer (Appendix 2). Haplotype A was widespread in all three *Fuscospora* species, being present in the North Island sites and 14 of the South Island sites. However it was less common in the south of the South Island. Haplotype B had a single T inserted in the repeating sequence. It was also shared by all three species, and was present in 7 South Island sites, all in the south-west of the island (Fig. 7.5). The sites were: F6, F7 (*N. fusca*), S7, S8, S9, S10 (*N. solandri* var. *cliffortioides*) and T4 (2 individuals, both *N. truncata*). The two *N. truncata* individuals at T4 were, prior to transplanting, within one or two metres of each other, and therefore probably had the same maternal parent (P. Garnock-Jones pers. comm.). B is not the exclusive haplotype in the south-west of the South Island, although appears to be more abundant than A.

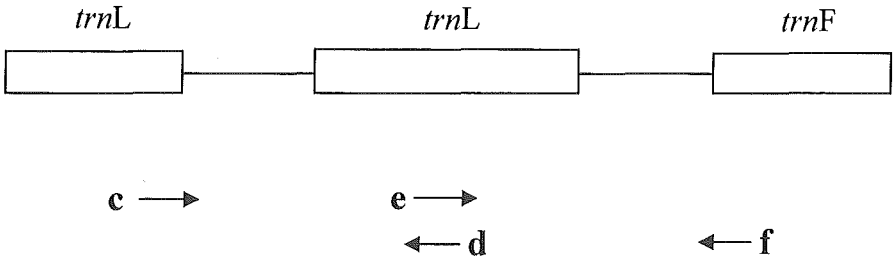

Fig. 7.3 Universal primers in the *trn*L and *trn*F genes, designed by Taberlet et al. (1991). Tips of arrows indicate the 3' ends of the primers. Primers 'c' and 'd' enclose the *trn*L intron, and primers 'e' and 'f' enclose the *trn*L-*trn*F intergenic spacer.

Based on Fig. 1 in Taberlet et al. (1991)

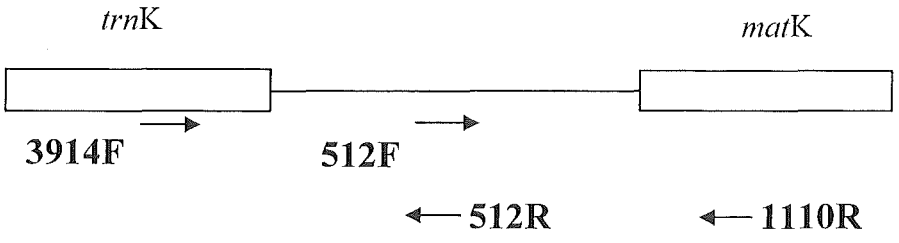

Fig. 7.4 Primers used for sequencing reactions for the *trn*K-*mat*K intergenic spacer. Tips of arrows indicate the 3' ends of the primers. *trn*K3914F and *mat*K1110R are the external primers and enclose the spacer. The internal primers, 512F and 512R, anneal within the spacer. These two primers were designed by S. Wagstaff, Landcare Research, Lincoln.

Table 7.2 Sequencing of *Fuscospora* samples across the *trn*L (Taberlet 'c') - *trn*F (Taberlet 'f') region. Refer Fig. 7.3 for primer positions.

Sample	Species	Start Position	End Position	Taberlet Primer(s)
F1	*N. fusca*	34	915	c,f
F2	*N. fusca*	42	915	c,f
F3	*N. fusca*	1	943	c,d,e,f
F4	*N. fusca*	42	915	c,f
F5	*N. fusca*	121	943	c,e,f
F6	*N. fusca*	33	916	c,f
F7	*N. fusca*	29	916	c,f
F8	*N. fusca*	531	943	e
F9-a	*N. fusca*	1	943	c,d,e,f
F9-b	*N. fusca*	528	943	e
S1	*N. solandri*	29	913	c,f
S2	*N. solandri*	529	943	e
S3	*N. solandri*	24	913	c,f
S4	*N. solandri*	120	943	c,e,f
S5	*N. solandri*	546	943	e
S6	*N. solandri*	545	943	e
S7	*N. solandri*	29	909	c,f
S8	*N. solandri*	29	914	c,f
S9	*N. solandri*	1	944	c,d,e,f
S10	*N. solandri*	1	944	c,d,e,f
T1	*N. truncata*	548	906	e
T2	*N. truncata*	534	943	e
T3	*N. truncata*	545	943	e
T4-a	*N. truncata*	48	904	c,f
T4-b	*N. truncata*	531	944	e

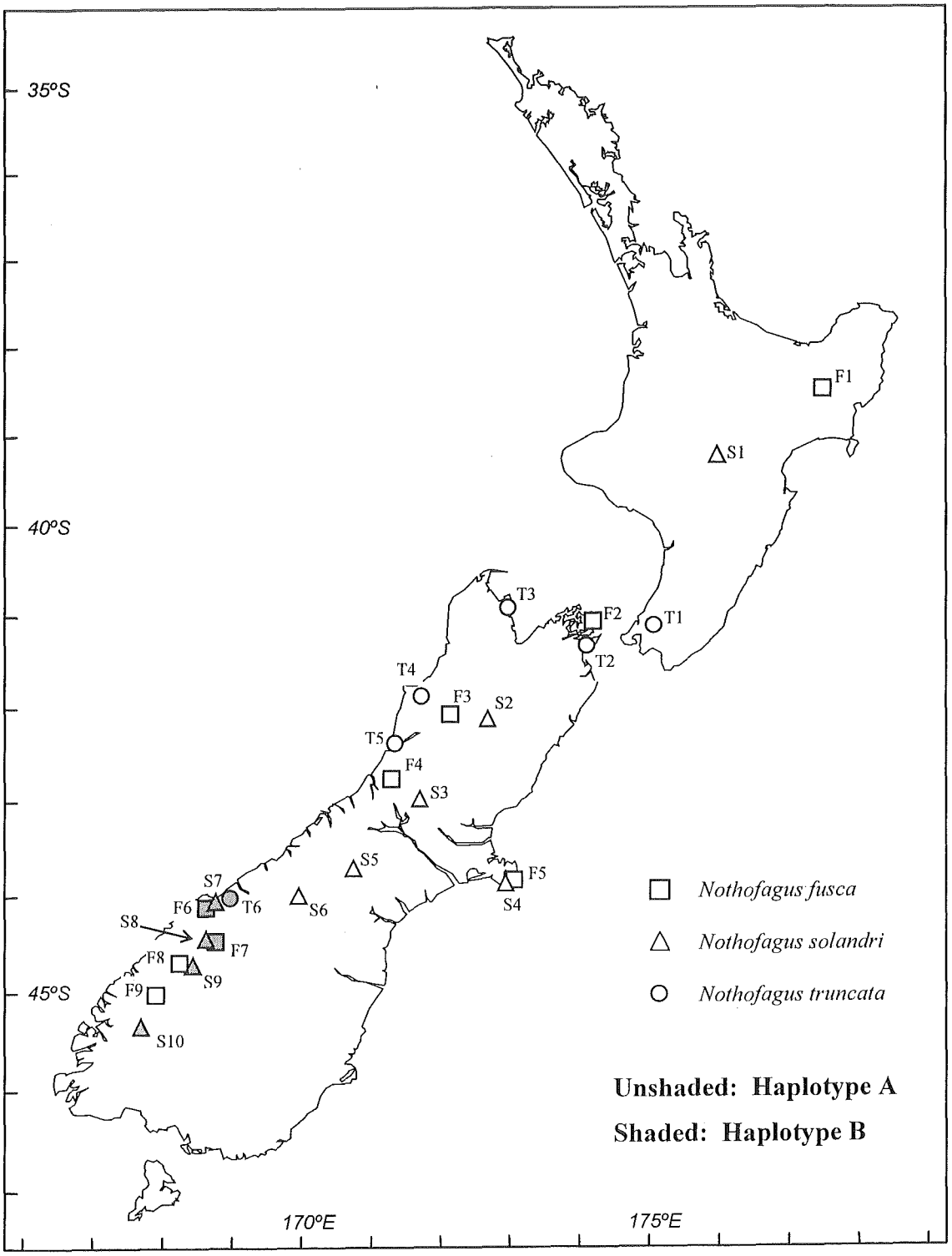

Fig 7.5 The two cpDNA haplotypes for *Nothofagus fusca*, *N. solandri* var. *cliffortioides* and *N. truncata*, as revealed by sequencing of the *trn*L-*trn*F intergenic region.

2. *trn*E - *trn*T

Initial sequencing reactions, utilising the *trn*E and *trn*T primers, were used to produce sequences across the full region for F3, F9, S9 and S10 (Table 7.3). Sequencing revealed no polymorphisms in this cpDNA region (Appendix 2).

Table 7.3 Sequencing of *Fuscospora* samples across the *trn*E - *trn*T region.

Sample	Species	Start Position	End Position
F3	*N. fusca*	1	522
F9	*N. fusca*	1	522
S9	*N. solandri*	1	522
S10	*N. solandri*	1	522

3. *trn*F - *ndh*J

Initial sequencing reactions, utilising the *trn*F and *ndh*J primers, were used to produce sequences across all or most of the region for F3, F9 and S9 (Table 7.4). Sequencing revealed no polymorphisms in this cpDNA region (Appendix 2).

Table 7.4 Sequencing of *Fuscospora* samples across the *trn*F - *ndh*J region.

Sample	Species	Start Position	End Position
F3	*N. fusca*	1	629
F9	*N. fusca*	1	644
S9	*N. solandri*	1	660

4. *trn*K-*mat*K

Initial sequencing reactions, utilising two external and two internal primers (Fig. 7.4), were used to produce sequences across all or most of this cpDNA region for F3, F9, S9 and S10. The two external primers, *trn*K3914F and *mat*K1110R are the

amplification primers (10) and (11) respectively (Table 6.4). The sequences (5' - 3') for internal primers 512F and 512R are AATCAAAAGAGCGATTGGGT and ACCCAATCGCTCTTTTGATT respectively.

Sequencing revealed no polymorphisms in this cpDNA region (Appendix 2).

Table 7.5 Sequencing of *Fuscospora* samples across the *trn*K - *mat*K region.

Sample	Species	Start Position	End Position
F3	*N. fusca*	1	951
F9	*N. fusca*	1	951
S9	*N. solandri*	1	951
S10	*N. solandri*	50	951

7.4 RFLP of cpDNA regions

The single polymorphism revealed from sequences of four cpDNA regions for four individuals did not fall within a recognition sequence for any of the restriction enzymes mapped by Sequencher v3.0 (Section 6.5.1). Therefore RFLP assays would not be informative in determining the distribution of this particular polymorphism.

It is possible, however, that assaying the four cpDNA regions across a fuller geographic range may reveal other polymorphisms not revealed by sequencing of the four individuals. Therefore trial RFLP assays were conducted upon nine individuals of *Nothofagus fusca* and *N. solandri* var. *cliffortioides*, from well-spaced sites within the South Island. If any further polymorphisms were to be detected, further assays would be conducted for that enzyme and cpDNA region across the full range of species and sites collected from.

For the trial assays, restriction enzymes for each cpDNA region were selected on their predicted utility in terms of number and size of fragments (Section 6.5.1), and on their ready availability.

Individuals from the following sites were tested:

- F2 - *Nothofagus fusca*, Marlborough Sounds
- F3 - *N. fusca*, Maruia Valley
- F4 - *N. fusca*, Otira Valley
- F6 - *N. fusca*, Jackson Bay
- F7 - *N. fusca*, East Matukituki
- F9 - *N. fusca*, Upper Eglinton
- S7 - *N. solandri* var. *cliffortioides*, Jackson Bay
- S8 - *N. solandri* var. *cliffortioides*, West Matukituki
- S10 - *N. solandri* var. *cliffortioides*, Te Anau

Only a small proportion of the amplified cpDNA regions could be assayed by the restriction enzymes used. In total, 14 digests produced fragments of the original cpDNA regions (Table 7.6), although only fragments of >100 base pairs were distinct enough to be considered informative. No polymorphisms were revealed (eg Fig. 7.6). The number of DNA base pairs in a cpDNA region that were assayed by a given enzyme was determined by multiplying the number of informative cutting sites by the length of the recognition sequence ('Total assayed DNA', Table 7.6). Totalling for all enzymes provides the total number of DNA base pairs in the cpDNA region assayed using RFLP ('Assayed DNA', Table 7.7). For no region was the proportion of DNA assayed greater than 5.1%.

Given that sequencing revealed only a single indel in the 2700 non-coding base pairs across all four regions, the lack of polymorphisms detected via RFLP trials is not surprising. Even if all of the potentially informative enzymes (producing multiple fragments of >50 base pairs each) indicated by Sequencher were utilised ('Potentially assayable DNA', Table 7.7), no more than 17.4% of the DNA in any of the cpDNA regions would be assayed.

Given the low probability of that proportion encompassing a mutation which had been undetected by sequencing (Section 7.3), RFLP was not persisted with following the initial trial.

Table 7.6 Successful restriction digests for four cpDNA regions. Fragments <100 base pairs were too indistinct to be informative. Total assayed DNA is no. of cutting sites multiplied by recognition sequence length.

cpDNA Region	**Restriction Enzyme**	**Fragment Sizes (base pairs)**	**No. of cutting sites**	**Recognition sequence length (base pairs)**	**Total assayed DNA (base pairs)**
*trn*F-*trn*L	*Hinf*I	420, 150, 120, 120 *	3	5	15
	*Dde*I	360, 280, 180, 100	3	5	15
	*Hae*III	520, 300	1	4	4
	*Rsa*I	480, 320, 200	2	4	8
	*Sal*I	450, 450 *	1	6	6
*trn*E-*trn*T	*Hae*III	300, 250	1	4	4
	*Hinf*I	340, 110	1	5	5
*trn*F-*ndh*J	*Eco*RI	400, 200	1	6	6
	*Hinf*I	250, 250 *	1	5	5
	*Hae*III	600, 150	1	4	4
	*Rsa*I	420, 150, 110	2	4	8
*trn*K-*mat*K	*Hinf*I	350, 250, 200, 100	3	5	15
	*Hae*II	560, 400	1	6	6
	*Rsa*I	680, 280	1	4	4

* Assumed to be two fragments of same approximate length, as predicted by Sequencher.

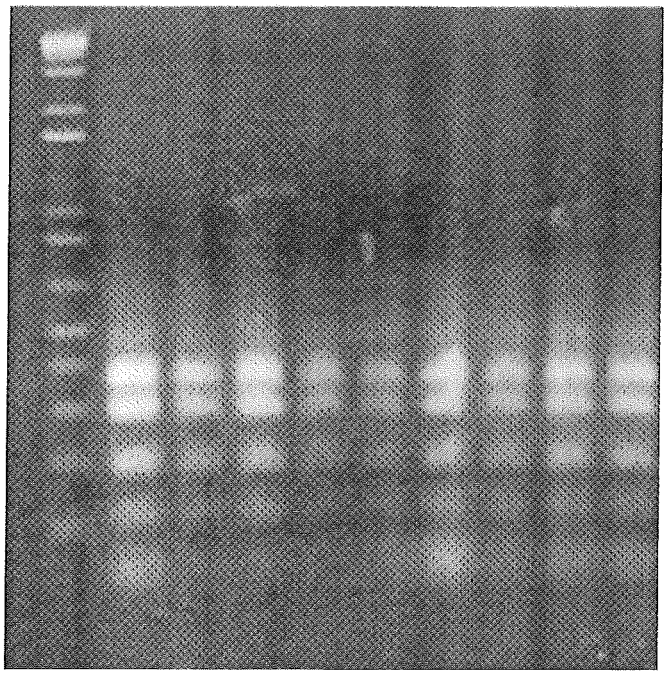

Fig. 7.6

Restriction patterns observed for the *trn*L-*trn*F cpDNA region digested with *Dde*I.
Lane 1 is 1 Kb Plus Ladder. Lanes 2-10 are F9, F7, F6, F4, F2, F3, S10, S8 and S7.

Table 7.7 Proportion of DNA assayed by RFLP in four cpDNA regions. 'Assayed DNA' is DNA assayed in this study (from Table 7.6). 'Potentially Assayable DNA' is what could be assayed if all potentially useful enzymes indicated by Sequencher were used.

cpDNA region	**Region length (base pairs)**	**Assayed DNA**		**Potentially assayable DNA**	
		Total (base pairs)	**% of region**	**Total (base pairs)**	**% of region**
*trn*L-*trn*F	943	48	5.1%	150	15.9%
*trn*E-*trn*T	522	9	1.7%	80	15.3%
*trn*F-*ndh*J	660	23	3.5%	115	17.4%
*trn*K-*mat*K	951	25	2.6%	155	16.3%

8 Discussion

8.1 Low variability of cpDNA in *Fuscospora*

The results of this study suggest that there is low variability in DNA sequences in the chloroplast genome in New Zealand *Fuscospora*. Of the four cpDNA regions sequenced, only one revealed any sequence variation among *Nothofagus fusca* and *N. solandri* var. *cliffortioides* samples. This one region (*trn*L-*trn*F) was comprehensively sampled from 22 sites across the South Island (Fig. 7.5). The other three cpDNA regions (*trn*F-*ndh*J, *trn*E-*trn*T, *trn*K-*mat*K), however, were sequenced from only three or four sites. Since that represents only a small part of their geographic distribution, it is possible that variability in these cpDNA regions has not been detected, despite sites being on opposite sides of the disjunction and representing two species.

The degree of evolutionary change in the non-coding cpDNA looked at in this study can be determined given (i) the total number of non-coding nucleotides sequenced, and (ii) the total number of mutations. The total number of nucleotides of non-coding DNA is slightly less than the total length of DNA sequenced (3076 base pairs), because of genes within the cpDNA regions. In the fully sequenced chloroplast genome for *Lotus japonicus* (Fabaceae) (Section 6.3.1), there are 319 base pairs of coding DNA within the four cpDNA regions. Assuming that *Fuscospora* has a very similar chloroplast genetic structure, this leaves around 2757 base pairs of non-coding DNA.

Where the number of occurences of insertions and deletions is small (as it is here), the degree of evolutionary change (g_m) is measured as the number of nucleotide insertions / deletions per site, and is

$$g_m = m_g / m_T \quad \text{(Nei 1987)}$$

where m_g is the number of nucleotides encompassed in all indels and m_T is the total number of nucleotides compared. By this measure, the degree of nucleotide insertion / deletion per nucleotide site in non-coding cpDNA in this study is calculated as 1 / 2757

or 0.00036. However this estimate cannot be considered reliable given the high degree of error associated with single stochastic events.

There is only one other known intraspecific sequencing study of cpDNA in *Nothofagus*, against which these rates of evolution can be compared. In *N. menziesii*, two substitutions were found in the *trn*L-*trn*F region for 29 individuals sampled across their distributional range in New Zealand (Stoeckler 2001). Of the 943 base pairs in the *trn*L-*trn*F region, around 95 base pairs are coding nucleotides (based on *Lotus japonicus*) leaving around 848 non-coding base pairs. Thus the degree of variability is 2/848 or 0.0024 substitutions per non-coding site, which is about 7 times greater than for *Fuscospora* in the present study. However a direct comparison is confounded by the stochastic nature of very low numbers of mutations in both cases, the different amount of non-coding DNA sampled, and the different sizes of geographic range from which samples were taken.

Two phylogenetic studies of *Nothofagus* based on cpDNA sequences also suggest that variation within and among the New Zealand *Fuscospora* is low. In sequences of around 953 base pairs of the *atp*B-*rbc*L intergenic spacer and 146 base pairs of the *rbc*L gene, the degree of variation is in line with the present study. The three New Zealand *Fuscospora* species differed amongst each other with substitutions at three nucleotide sites (Setoguchi et al. 1997). In the second study, *N. truncata* differed from *N. fusca* and *N. solandri* by a surprisingly high 9 substitutions in sequences from the *rbc*L gene only (Martin and Dowd 1993). Although the *N. truncata* used in that study was sourced from the isolated population in South Westland (P. Garnock-Jones pers. comm.), the variation is surprisingly high in this slowly evolving cpDNA region, even if the *N. fusca* and *N. solandri* samples came from distant provenances elsewhere in New Zealand. The most plausible explanation is that error rates were higher in early manual sequencing procedures than with automatic sequencing (S. Wagstaff pers. comm.).

These results suggest that intraspecific cpDNA variation is low in the New Zealand *Fuscospora* (and possibly in *Nothofagus* as a whole) compared to related forest tree genera. For example five substitutions and four indels were found within the *trn*L intron

and *trn*L-F spacer (together corresponding to the *trn*L-*trn*F region in this study) among *Fagus crenata* individuals collected from 21 populations across its distributional range in Japan. Even *F. japonica*, which was collected from only four populations, revealed five substitutions and two indels (Okaura and Harada 2002).

8.1.1 Reasons for low cpDNA variability

Possible reasons why cpDNA sequence variation is very low among the New Zealand *Fuscospora* individuals are: (i) very slow evolutionary rates in cpDNA in *Nothofagus*, (ii) loss of genetic variation through genetic bottlenecks, or (iii) very recent emergence of the *Fuscospora* complex.

In turn:

(i) There is some evidence that cpDNA in *Nothofagus* does evolve relatively slowly, in common with other tree species. In the *rbc*L gene, *Nothofagus* taxa have had an average 0.015 ± 0.006 substitutions per nucleotide site in the last 80 million years (Manen et al. 1998), translating to 0.187×10^{-9} substitutions/site/year. This is slightly higher than for other tree genera, for example the 0.071×10^{-9} substitutions/site/year calculated for *Castanea* (Fagaceae) (Frascaria et al. 1993) and the 0.089×10^{-9} substitutions/site/year for *Ilex* (Aquifoliaceae) (Manen et al. 1998). All of these rates are significantly lower than for predominantly non-tree lineages such as the tribe Rubieae (Rubiaceae) (2.10×10^{-9} substitutions/site/year) (Manen et al. 1998) and Asteraceae (0.569×10^{-9} substitutions/site/year) (Bremer and Gustafsson 1997).

(ii) Even by the standards of *Nothofagus*, variation within and among the New Zealand species of *Fuscospora* may be particularly low. Geological or climatic events may have restricted all of the *Fuscospora* species, or their common ancestor, to small refugia, and reduced genetic diversity within the lineage. Some poorly-dispersed New Zealand taxa may be particularly

vulnerable to genetic bottlenecks, given the size of the landmass, and the absence of migration routes. Pollen evidence suggests that there were times when *Fuscospora* was scarce in New Zealand, for example during parts of the Miocene (McGlone et al. 1996). A genetic bottleneck may also have occurred during the Oligocene, when only a very small proportion of the New Zealand landmass remained above sea level (McGlone 1985). The low variation may also be due to founder effects. The divergence of the New Zealand *Fuscospora* from their closest relative, *N. gunnii,* has been estimated at 16 mya based on nuclear ITS sequences and 11 mya based on the *trn*L intron and *trn*L-*trn*F intergenic spacer region (K. Stoekler unpubl. thesis). This may post-date some of the above events; if these species evolved as a consequence of long-distance dispersal from the ancestor of *N. gunnii*, then they may have arisen from a very small number of colonising propagules.

(iii) Dating the divergence of the three New Zealand *Fuscospora* species is difficult due to the paucity of macrofossil evidence. The most common fossil evidence is pollen, but pollen of the three species is indistinguishable. If the common ancestor of the New Zealand *Fuscospora* diverged from *Nothofagus gunnii* 11 mya (Stoeckler 2001), then their divergence from each other has occurred since then.

8.2 Chloroplast sharing among New Zealand *Fuscospora* species

The sharing of chloroplast haplotypes in the New Zealand *Fuscospora*, by spatial distribution, rather than along phylogenetic lines, is not unique. As early as 1991, 37 examples of chloroplast introgression had been published (Rieseberg and Wendel 1993). It has been documented in some angiosperm tree genera including *Quercus* (Whittemore and Schaal 1991; Ferris et al. 1993; Petit et al. 1997), *Alnus* (King and Ferris 2000) and *Eucalyptus* (Steane et al. 1998; McKinnon et al. 1999, 2001).

One implication of this finding is that the phylogenetic relationship between the three New Zealand *Fuscospora* cannot be resolved on the basis of chloroplast DNA. This is supported by other *Nothofagus* molecular studies using cpDNA, which have not been informative on the phylogenetic relationship of these three species. The three species formed unresolved trichotomies in a strict minimal consensus tree based on *rbc*L sequences (Martin and Dowd 1993), and in a maximum likelihood analysis based on *trn*L intron and *trn*L-*trn*F sequences (Stoeckler 2001). Clearly, inferring any dichotomous phylogeny would be risky given that the cpDNA sequence for an individual is determined more by the geographical location where it was sampled than by its species.

On the other hand, stronger phylogenetic resolution has been achieved in strict consensus trees using a combination of morphological and nuclear data. These support a (*N. solandri* - (*N. fusca* - *N. truncata*)) grouping (Manos 1997).

The possibility that the shared cpDNA mutation is caused by parallel evolution is very remote. From our understanding of the slow rate of *Nothofagus* molecular evolution, a single mutation in non-coding chloroplast DNA is a rare event. For the same neutral evolutionary event to occur independently in each of the three species within the same restricted geographical area is unlikely enough to be discounted.

The most popular explanation is that genetic exchange has occurred in the form of introgression (Rieseberg and Brunsfeld 1992). Introgression is the incorporation of a gene from one population pool, be it a single population, subspecies or species, into

another population pool. It has an element of permanence, to distinguish it from the genetic exchanges that occur in hybrid swarms (Rieseberg and Wendel 1993). Molecular techniques have demonstrated that introgression occurs far more commonly than was previously suspected from morphological analysis alone.

8.2.1 Hybridisation among *Fuscospora* species

If introgression of chloroplast genotypes among species has occurred, then this depends on hybridisation occuring among those species. This implies that the species must be, or have been, sympatric or at least geographically close enough for cross-fertilisation to occur. The hybrids should be fertile or partially fertile, allowing backcrossing to at least one of the parent species.

All of these criteria appear to apply to the three New Zealand *Fuscospora* species, all three of which hybridise naturally with each other. Although there is a lack of experimental or observational data on the fertility of F1 and subsequent hybrids relative to parental species, there is some evidence that F1 hybrids are able to backcross. The high variability of hybrid forms in some areas have been interpreted as indicating the presence of hybrid swarms (Wardle et al. 1988). Given that F1 hybrids can be relatively uniform (Anderson 1948; Stebbins 1950), hybrid swarms suggest that F1 hybrids are fertile. This is supported by observed open pollination of putative *N. fusca* X *N. solandri* var. *cliffortioides* F1 hybrids, resulting in offspring with highly variable phenotypes, including forms approaching the two parental species (Poole 1951).

Hybridisation among *Fuscospora* occurs most commonly where species ranges overlap (Wardle 1984; Wardle et al. 1988). The frequency of hybridisation tends to be limited by the strength of habitat preferences of the parent species (Anderson 1948; Rieseberg and Wendel 1993). Hybridisation is most common where these barriers break down and competition is not so strong, such as where disturbance has occurred. It also occurs where overlapping species boundaries are changing due to climatic fluctuation (Anderson 1948). In the New Zealand *Fuscospora*, high hybridisation rates have been observed in

disturbed forests (Wardle et al. 1988), and in forests where *N. fusca* may be losing ground to *N. solandri* var. *cliffortioides* (Holloway 1954).

It is likely that the South Island has provided abundant settings for hybridisation during the Pleistocene. Globally, hybridisation was probably frequent, and played a role in the spread of flora into lands denuded by the Pleistocene glaciations (Anderson 1948). Disturbances in the South Island were (and continue to be) frequent due to tectonic uplift and erosion (Section 3.1.2). Cooling and warming episodes caused marked changes in species boundaries, as forest trees reoccupied former glacial and open habitats, or underwent habitat shifts following temperature fluctuations.

Hybridisation rates do not need to be high for introgression of chloroplast genomes to occur. For example, there is evidence for high cpDNA gene flow between North American *Quercus* species, in spite of very little sign of hybridisation in the field (Whittemore and Schaal 1991). It is however possible that hybridisation was more common in the past, when species were expanding their ranges following the last glacial period.

8.2.2 Causes of introgression between species

Introgression may arise where one hybridising parent greatly outnumbers the other, resulting in an asymmetrical cpDNA flow, from the dominant species to the scarce one (Rieseberg and Wendel 1993). Species imbalance still applies in the south of the South Island, for *N. truncata* at least, and was probably even more pronounced during the glacial maxima and early Holocene. If all three species survived in the south during the glacial maxima, *N. solandri* var. *cliffortioides* was probably overwhelmingly more abundant than *N. fusca* and *N. truncata*, which must have been very rare and localised. Alternatively, if the latter two species dispersed there during the Holocene, the original founding populations would have been greatly outnumbered by *N. solandri* var. *cliffortioides*.

A further possibility is asymmetric gene flow due to unidirectional hybridisation. Asymmetric gene flow can be caused by 'unilateral cross-incompatibility', observed among species with markedly different floral morphology, particularly in terms of relative stigma lengths – thus pollen tubes of one species may successfully grow down the style of the other species, but not vice versa (Gore et al. 1990). Alternatively, asymmetry could be due to phenological reasons, such as synchronisation of flowering times (eg synchronisation of pollen release in one species and female receptiveness in the other). Asymmetrical hybridisation has also been attributed to genetic incompatibility. In a hybrid swarm in *Populus*, all hybrids observed were either F1 hybrids or backcrosses to one of the parent species only. Backcrosses to the other parent species were thought to fail due to genetic barriers to reproduction causing aborted seed or abnormally developing seed (Keim et al. 1989).

In the absence of any significant studies into the relative hybridisation success and fertility of *Fuscospora* F1 hybrids and respective backcrosses, the causes and direction of introgression can only be guessed at. If asymmetrical hybridisation were revealed, contributing factors could be phenological, morphological or genetic.

8.2.3 Possible chloroplast introgression in the absence of nuclear introgression

Although chloroplast genetic exchange has been observed in this study, there is a possibility that this has not been accompanied by substantial nuclear exchange. There is no documented evidence of introgression based on morphological studies. There have been only very limited direct comparisons of *Fuscospora* nuclear genomes (Haase 1992, 1994). Allozyme analysis suggested that *N. solandri* var. *cliffortioides* in south Westland was as genetically distant from *N. truncata* in this location as it was from *N. truncata* elsewhere in New Zealand. Haase interpreted this as there being no evidence of gene flow (by which he presumably meant no nuclear gene flow) between the two in South Westland, although this conclusion was based on sampling of single populations. A separate, and again small, allozyme analysis of the three *Fuscospora* species, mostly in

the northern half of the South Island, did not suggest any gene flow among *Fuscospora* species at various sites in the South Island (Haase 1994).

The incongruence observed between nuclear introgression (Haase's study) and chloroplast introgression (this study), if correct, would not be surprising. Cytoplasmic introgression has often been detected in taxa in which introgression had previously been rejected on morphological and nuclear evidence (Rieseberg & Wendel 1993). Rieseberg and Soltis (1991) summarise instances in a diverse range of taxa (including *Quercus*, *Helianthus*, *Gossypium*, *Populus* and *Zea*) where cytoplasmic introgression in plants appears to have occurred in the absence of significant nuclear exchange.

The disassociation between nuclear and cytoplasmic gene flow is a direct result of genetic movement in plants being either via pollen or seeds. In most angiosperms, seeds transmit both nuclear and cytoplasmic genomes, whilst pollen transmits nuclear genomes only. The result of an interspecific hybridisation is that the offspring has a nuclear genotype contributed by both parents, but cytoplasmic genomes contributed by the maternal parent only.

Again, in the absence of empirical data on hybridisation among these species, the causes of any incongruence between nuclear and cytoplasmic gene flow in the New Zealand *Fuscospora*, can only be speculation. The phenomenon may be caused by a differential in selection forces on cpDNA and nuclear genes. Thus, in an introgressed individual, selection may act against linked foreign nuclear genes, but not against cytoplasmic genes. If some nuclear genes are strongly selected against following hybridisation, then linkage may ensure that the remainder of the nuclear genome does not introgress either. On the other hand, cytoplasmic DNA is not linked to nuclear genes, and is therefore not indirectly subject to strong selection (Barton and Jones 1983; Rieseberg and Wendel 1993).

Alternatively, unidirectional hybridisation (Section 8.2.2) may cause cytoplasmic DNA to be transmitted in the absence of nuclear gene transfer. This would occur when hybrid backcrosses only occur in the direction of the pollen parent species. Thus the cytoplasm of the maternal parent species could be ultimately introgressed into the pollen parent

species. A further, and related, explanation could be the widespread phenomenon of 'cytoplasmic male sterility' (CMS). The pollen of F1 hybrids is rendered sterile as a result of incompatibility between the uniparentally-inherited mtDNA and biparentally-inherited nuclear DNA. Backcrosses to the original pollen-donor parent will therefore only be via female propagules, and will thus have the cytoplasmic genome from the original female parent. Further backcrossing ultimately leads to the introgression of cytoplasm into that population (Rieseberg and Wendel 1993; Avise 1994).

8.3 Evaluation of historical distributions and gene flow in the South Island

Resolution of a number of haplotypes would have permitted the reconstruction of a phylogeny, which, when overlain with present-day distributions, may have allowed inferences on pleistocene refugia and migrational pathways in the Holocene to be made. Although only two haplotypes (arising from a single polymorphism) were detected in this study (Fig. 7.5), the spatial distribution of these haplotypes, and the occurrence of chloroplast sharing, does allow some inferences to be made.

A differentiation in haplotype frequencies north and south of the disjunction is apparent from visual inspection, and can easily be tested statistically, using a χ^2 test. If the *Fuscospora* disjunction to the east of the main divide is taken to be between the Rangitata valley and Lake Pukaki, then 7 of the 10 sites south of the disjunction have haplotype B, whereas none of the 12 sites in the South Island north of the disjunction possess this haplotype. The hypothesis H_O, that the frequency of haplotype B is independent of whether north or south of the disjunction, is tested against H_A, that the frequency of haplotype B depends on location. From the 2 x 2 contingency table (Table 8.1), χ^2 = 12.31, DF = 1 and P = 0.000451. Therefore H_O, that the haplotype frequency is independent of location is rejected, and a differential in haplotype frequencies on either side of the disjunction is strongly supported.

8.3.1 Origin and spread of the mutation

The ancestral haplotype can be determined by reference to the closest relative to the New Zealand *Fuscospora*, *Nothofagus gunnii*, a deciduous shrub found only in Tasmania. At the site of the single polymorphism found in this study, positions 838-844 in the *trn*L intron and *trn*L-*trn*F intergenic region (Section 7.3), *N. gunnii* shares the same character as haplotype A (Stoeckler 2001). Haplotype A is therefore inferred to be ancestral, and the mutation distinguishing haplotype B occurred since divergence of *Fuscospora* from *N. gunnii*.

Table 8.1 A 2 x 2 contingency table for testing the independence of *Fuscospora* haplotype frequency and location north or south of the South Island disjunction. H_O = haplotype frequency is independent of location. H_A = haplotype frequency is not independent of location. Each cell has observed frequency, with expected frequency if H_O is true in parentheses.

	Haplotype Frequency		
Side of Disjunction	A	B	Total
North	12 (8.182)	0 (3.818)	12
South	3 (6.818)	7 (3.182)	10
Total	15	7	22

A very approximate estimate of the date of origin of the mutation can again be made by comparison with *N. gunnii*. However the stochastic nature of a single mutation makes any estimate very approximate. Within the *trn*L intron and *trn*L-*trn*F sequences, *N. gunnii* and the New Zealand *Fuscospora* differ by 4 base pairs. Assuming substitution rates of 0.235 +/- 0.039 x 10^{-9} substitutions/site/year in these cpDNA regions, *N. gunnii* and the New Zealand *Fuscospora* diverged 11 mya (Stoeckler 2001). In that time, therefore, 2 substitutions are expected to have become fixed in the New Zealand *Fuscospora*, followed by the mutation observed in this study achieving only a relatively localised distribution. This mutation therefore probably occurred no more than 1-2 mya, and quite possibly within the last million years. However, given the slow evolutionary rate of non-coding cpDNA in *Nothofagus*, it appears unlikely that the mutation arose during the Holocene.

The range of the observed mutation can be compared with that of a unique 'southern South Island' haplotype observed in *Nothofagus menziesii* from sequences in the *trn*L

intron and *trn*L-*trn*F intergenic region (Stoeckler 2001). All 8 samples from the southern half of the island carried this haplotype: five of these samples came from the same general area (Catlins), although the other three (Mt Cook, Haast, Longwood Range) were widely spaced. This haplotype differs from that in the northern half of the South Island by a single substitution within the *trn*L intron. The haplotype in the northern South Island is probably ancestral (Stoeckler 2001), and the confinement of the 'southern South Island' haplotype suggests that seed-mediated dispersal has been limited, at least since the mutation occured.

The current distribution of the mutation in *Fuscospora*, and its confinement in the south of the South Island has probably been influenced by a number of factors:

1. Stochastic environmental events. If we assume that the allele is selectively neutral, then increases and decreases in its distribution and frequency over time are dictated by genetic drift and stochastic episodes such as bottlenecking. Genetic bottlenecking can bring about relatively abrupt increases or decreases in the incidence of a random allele. The geological upheavals and climatic fluctuations in the South Island during the late Tertiary and Quaternary were a likely setting for bottlenecking in *Nothofagus* and other South Island taxa. For example, repeated bottlenecking probably occurred during the numerous glacial periods of the Pleistocene, when *Nothofagus* retreated to a small number of isolated refugia (Section 3.3.3).

2. Rates of spread. Given the assumption that the chloroplast genome is maternally inherited in *Nothofagus* (Section 4.3), its spread is mediated by seed dispersal. *Nothofagus* seeds normally do not disperse far, but may be dispersed across many kilometres by severe weather episodes (Section 3.2). Consequently, it is difficult to judge whether the observed geographical extent of haplotype B is due to rapid dispersal of a relatively recent mutation, or to slow dispersal of a far older mutation.

3. Barriers to spread. Apart from obvious features such as mountain ranges, barriers include habitats where *Nothofagus* is outcompeted by other vegetation types. Barriers were more pronounced during the glacial periods, when *Nothofagus* migration was restricted by ice cover and cold, exposed environments. In present-day central

Westland (and presumably during earlier interglacials), *Nothofagus* migration is either slowed (according to the glacial refugia hypothesis) or halted (according to the environmental barriers hypothesis) by competition with podocarps and broadleaf trees.

8.3.1.1 Selection not a likely factor

There is a theoretical possibility that selection is a factor in causing the spread and current distribution of haplotype B. Taken in isolation, mutations in non-coding cpDNA are selectively neutral. However, given the absence of recombination in the chloroplast genome, the observed mutation could have evolved in concert with mutations within genetic regions of the chloroplast genome. Consequently, this mutation may be 'hitchhiking' on positively-selectively chloroplast alleles

If the observed mutation were associated with a particular genotype with non-neutral alleles, then the mutation would be a marker for the distribution of this genotype. If the genotype confers a selective advantage in certain conditions, then its migration and range expansion may be more rapid than for a neutral genotype. For example, the genotype may be favoured by climates in the south of the island, and be actively spreading.

Having acknowledged that selection is a possible factor in structuring the distribution of *Fuscospora* chloroplast genotypes, it appears unlikely in this case. As already established, mutation rates in non-coding *Fuscospora* cpDNA are very low (Section 8.1). Rates in coding cpDNA are even lower, and synonymous substitutions are likely to form most of these. Therefore there is only a small likelihood of non-synonymous substitutions occuring in the chloroplast genome, particularly of such substitutions conferring any selective advantage.

8.3.2 Influences on haplotype distribution during the Pleistocene

Any attempt to reconstruct the history of haplotype distribution and frequency in the south of the South Island, is confused by the repeated glacial-interglacial cycles during

the Quaternary. Each cycle, if it conformed to the last glacial-interglacial, probably saw *Fuscospora* restricted to small isolated refugia during glacial periods, followed by expansion outwards from refugia and coalescence of populations during interglacials.

The main influences on the proportion of haplotypes in a given refugium were most likely the proportion of haplotypes of individuals at the location when the glacial period started, and the effects of genetic drift whilst the population was very small. If it is assumed that refugia existed in the same general locations during consecutive glacial periods, the contribution of haplotypes from other refugia during interglacials may not have been significant. This is because the migration rate of cpDNA haplotypes via seed dispersal was probably greatly limited after expanding populations coalesced.

8.3.3 Glacial refugia in the south of the South Island

The distribution of haplotypes suggests that glacial refugia may have been distributed in more than one location in the south of the South Island.

Haplotype B occurrences are at their highest proportion in South Westland and in the Matukituki valley. According to the criteria for glacial refugia (Section 3.4.2), the most likely refugia for *Fuscospora* in this area are among the low coastal hills south of Haast. During the Holocene, *Fuscospora* probably migrated up the South Westland valleys such as the Arawata and Waiototo. These valleys, being adjacent at the Main Divide, are the most likely migration route into the Matukituki valley which was entirely glaciated during the last glacial maximum (New Zealand Geological Survey 1973). All five individuals sequenced from the South Westland and Matukituki areas possess haplotype B, suggesting that this was the dominant, if not fixed, haplotype in the South Westland refugium or refugia.

The sampling sites in the Rees, Dart and Eglinton valleys were also glaciated during the last glacial maximum (New Zealand Geological Survey 1973). Post-glacial migration into these valleys may have originated from South Westland via valleys such as the

Hollyford, or from refugia on the south coast, from which there are no major barriers to migration. The mix of haplotypes from these sites suggests either:

- If migration has occurred from a single refugium, then that refugium had a mixture of haplotype A and B individuals at the end of the last glacial maximum, or
- If migration has occurred from more that one refugium, then the mixture represents a complex picture of coalescing populations, each with its own proportion of haplotype A and B individuals.

8.3.4 Post-glacial dispersal hypothesis not supported

The distribution of haplotype B strongly suggests that at least one species of *Fuscospora* survived in the south throughout the last glacial period, and possibly throughout the entire Pleistocene. The alternative hypothesis is that all *Fuscospora* species were eliminated from the south of the island during the last glacial period, and re-established via long distance seed dispersal during the Holocene. If this were the case, the existence and distribution of haplotype B can be explained in one of two ways, neither of which is probable:

1. *Fuscospora* dispersed south of the disjunction during the Holocene, after which the haplotype B mutation had time to arise and spread to its current extent. Given that in *Nothofagus*, average evolutionary rates in non-coding cpDNA are in the order of one mutation every several million years at least, this eventuality is remote (Section 8.3.1).
2. The haplotype B mutation exists infrequently to the north of the disjunction, and by chance was disproportionately represented in the few seeds dispersing to the south. Thus the mutation arose in the south from a founder effect. Given that none of the 12 samples in the South Island north of the disjunction uncovered the mutation in the north, there is a low probability of this.

8.3.5 Vicariance hypotheses evaluated

The very broad estimate for the time of origin of the southern South Island mutation (Section 8.3.1) is compatible with both the 'glacial refugia' and 'environmental barriers' hypotheses, but does not allow discrimination between either. The advent of the central South Island disjunction predated the origin of the mutation, if it is assumed that the mutation arose after the cessation of gene flow between the north and south of the island. The origin of haplotype B is estimated to be in the order of 1-2 mya at most, and is unlikely to have occurred as recently as the Holocene. Such a broad time span for the advent of the disjunction is compatible with scenarios associated with both of the glacial refugia and environmental barriers hypotheses (Section 3.1), and therefore neither can be discounted.

Both of these hypotheses are compatible with the current distribution of haplotype B, for which the rate of spread is subject to stochastic events (eg genetic bottlenecking), seed dispersal and the existence of barriers (Section 8.3.1). If the environmental barriers hypothesis were true, the disjunction may have been an effective barrier to the transmission of seed-mediated haplotype B from the south to the north of the South Island for up to several million years. Although, within this time frame, sporadic dispersal of the haplotype to the north cannot be dismissed, there is no evidence from this study that this has occurred.

For the glacial hypothesis, the picture is more complex, since during interglacial periods *Fuscospora* may have bridged the gap and populations from the north and south of the disjunction may have coalesced. This does not imply however, that haplotype B was able to migrate to the north of the disjunction. Although haplotype B may migrate north rapidly while *Fuscospora* displaces a podocarp-broadleaf forest, migration would be slowed following coalescence with northern *Fuscospora* in central Westland. Without selective pressure in its favour, there would appear to be a low likelihood of haplotype B progeny migrating a further 100-200 kilometres north to refugia in the north of the island prior to the next glacial period.

The estimated age of the mutation does not support the 'lateral plate shift' hypothesis. This predicts that genetic isolation may have existed for at least 20 million years (Section 3.1.3), and therefore for more mutations to have occured. A maximum value set on the age of the mutation, 2 million years, is only a tenth of what would be expected over such a period of isolation. Furthermore, if the ancestor of the New Zealand *Fuscospora* originated there as a result of dispersal from the *Nothofagus gunnii* ancestor in Australia 11-16 mya, this would postdate the putative population split by 5-10 million years.

8.3.6 Banks Peninsula disjunction

The small Banks Peninsula populations of *N. fusca* and *N. solandri* are themselves disjunct by a distance of around 75 kilometres from their nearest conspecifics. The sharing of haplotype A suggests that they have an affinity with populations north of, rather than south of, the disjunction. This is not surprising given the relative proximity of the former. Banks Peninsula was a volcanic island until becoming joined to the accreting Canterbury plains as recently as the last glacial period. A vicariance origin to the disjunction has been suggested by Wilson (1998) who proposed that *Fuscospora* migrated across the plains to the peninsula during the last glacial period, and was subsequently eliminated from the plains. However, given the inhospitable nature of the plains during the glacial period (Section 3.3.2), it is at least equally likely that *Fuscospora* migrated to the peninsula some time over its 10 million year history via long distance seed dispersal.

8.3.7 Evaluation of *N. fusca* / *N. truncata* survival south of the disjunction

Haplotype distributions in this study support the generally held view that at least one of the *Fuscospora* species survived in the south of the South Island during the last glacial period. The most likely to have survived is *Nothofagus solandri* var. *cliffortioides* (Section 3.5), and it is almost certain that some populations at least of this species in the south had the haplotype B mutation at the end of the last glacial period (Section 8.3.4).

If it is assumed that *N. solandri* var. *cliffortioides* did survive the last glacial period in the south of the island, then the three alternative hypotheses relating to the history of *N. fusca* and *N. truncata*, as summarised from section 3.5, are:

1) *N. fusca* and/or *N. truncata* survived in the south along with *N. solandri* var. *cliffortioides* during the last glacial period.

2) *N. fusca* and/or *N. truncata* failed to survive in the south during the last glacial period, and re-established there via long-distance seed dispersal.

3) *N. fusca* and/or *N. truncata* failed to survive in the south during the last glacial period, and re-established there via long-distance pollen dispersal and hybridisation.

All three of these are compatible with the patterns of chloroplast sharing found in this study. (1) and (2) will now be examined in the light of introgression as an explanation, whilst (3) will be detailed in section 8.3.8.

(1) *N. fusca* and/or *N. truncata* survival in the south

If all three of the New Zealand *Fuscospora* species have survived continuously in the far south of the South Island during the Pleistocene, then introgression may have commenced soon after the haplotype B mutation first appeared in one of the three species. If the mutation arose first in *Nothofagus solandri* var. *cliffortioides*, the numerical dominance of this species (particularly during the glacial periods) suggests that introgression may have proceeded from this species in the direction of *N. fusca* and *N. truncata* (Section 8.2.2). However origin of the mutation in *N. fusca* or *N. truncata*, and subsequent introgression into the far more abundant *N. solandri* var. *cliffortioides*, can not be ruled out. However this probably requires a factor such as unidirectional hybridisation (Section 8.2.2), which has not yet been observed in *Fuscospora*.

(2) Holocene *N. fusca* and/or *N. truncata* seed dispersal

Even with very recent establishment of *N. fusca* and *N. truncata* south of the disjunction via long-distance seed dispersal, introgression from *N. solandri* into these species may have had time to occur. This is because introgression of chloroplast genotypes of newly introduced species can happen very quickly, probably as a result of hybridisation and introgression occuring within founders. For example within fifty years of first appearing in southern California, almost all sampled individuals of the annual *Helianthus petiolaris* shared their chloroplast genome with sympatric individuals of its close relative, *H. annuus* (Dorado et al. 1992). If a generation time of one year is assumed, the 50 generations taken to achieve this is not dissimilar to the number of generations of *Nothofagus* during the Holocene, if a single generation of *Nothofagus* is assumed to be 100-200 years. Some North American *Quercus* species have shared regional chloroplast haplotypes, in spite of having only migrated to their present locations in the ~100 generations possible during the Holocene (Whittemore and Schaal 1991).

8.3.8 Long-distance pollen dispersal and hybridisation hypothesis

The results of this study suggest that the long-distance pollen hybridisation hypothesis (Wardle et al. 1988; Section 3.5)) cannot be rejected. The scenario predicts that the reconstituted *N. fusca* and *N. truncata* to the south of the disjunction would share their maternally-inherited chloroplast genome with the progenitor of the female line, *N. solandri* var. *cliffortioides*.

The idea that species dispersal can be effected by pollen flow and hybridisation, rather than seed dispersal, has been advanced from morphological (Potts 1986; Potts and Reid 1988) and cpDNA studies (Petit et al. 1997). The idea is particularly applicable to plant lineages with high pollen dispersal but low seed dispersal. Potts and Reid (1988) described patches of intermediate *Eucalpytus risdonii* and *E. amygdalina* phenotypes in *E. amygdalina* forest, 200-300 metres from a *E. risdonii* boundary, well beyond the normal seed dispersal distance of *E. risdonii*. The patches are in favourable *E. risdonii* habitat, and are considered to be founded by F1 hybrids which are the oldest in the patch.

Some patches are dominated by *E. risdonii*-type hybrids, which arose either by (i) in the presence of *E. risdonii* pollen, backcrossing of the F1 hybrids to *E. risdonii*, (ii) self-pollination or crossing with other F1 hybrids in the same patch, which would produce highly variable offspring. In favourable *E. risdonii* sites, *E. risdonii*-type phenotypes would be favoured, and in subsequent generations, even with minimal further *E. risdonii* pollen input, *E. risdonii* phenotypes would segregate out.

A second example is that of the sympatric species pair, *Quercus robur* and *Q. petraea.* Petit et al. (1997) found that geographic patterns for cpDNA variation consisted of large patches, and that both species usually shared the same cpDNA haplotype within a patch. Given the low dispersal of acorns compared to pollen, they concluded that the most parsimonious explanation for post-glacial migration was as follows: The pioneer species, *Q. robur*, colonised new patches; hybridisation occurred as a result of pollen flow from distant shade-tolerant *Q. petraea*; in the understorey, backcrosses to *Q. petraea* were favoured, and ultimately led to a 'swamping' of the *Q. robur* nuclear genome.

Since the nuclear genome of the maternal species in these examples is progressively replaced by that of the pollen parent, dissimilarity between the nuclear genomes of the maternal parent and the reconstituted forms is not sufficient to discount this hypothesis. This throws into question the conclusion drawn by Haase (1992), who dismissed the hypothesis on the basis of allozyme dissimilarity between *N. solandri* var. *cliffortioides* and *N. truncata* in south Westland.

The hypothesis depends on the dispersal and fertilisation by *N. fusca* and *N. truncata* pollen from distances of at least 300 km. This is possible, since *Fuscospora* pollen is known to disperse by wind over distances up to hundreds of kilometres at least. It has appeared, for example, in pollen counts in the Chatham Islands where *Nothofagus* is absent, around 800 kilometres east of the South Island (McGlone 1988). Even allowing for a relatively low wind speed of 10 km/hr, Wardle et al. (1988) concluded that up to 40% of pollen would still be viable in the 30 hours needed to disperse across the disjunction. *N. fusca* x *N. solandri* hybrids have been found in a *N. solandri* var. *cliffortioides* stand, 2 km from the nearest *N. fusca* pollen source, while hybrids in

another stand may be up to 40 km from the nearest *N. fusca* source (Wardle et al. 1988). In several other New Zealand genera, hybrids have been observed hundreds of kilometres beyond the natural range of the pollen parent (Wardle et al. 1988).

For F1 hybrids to produce offspring phenotypes approaching those of the respective pollen parents, the hypothesis depends on either (i) backcrossing of the F1 hybrids to pollen parents, or (ii) crossing with other F1 hybrids to produce a wide range of F2 phenotypes, some of which will resemble those of the pollen parent (Wardle et al. 1988). Either is feasible given that fertility of *Fuscospora* F1 hybrids and of backcrosses is assumed (Section 8.2.1), although scenario (ii) is more likely if F1 hybrids are at all partially sterile. Selection will then favour phenotypes most resembling the pollen parent if they are already adapted to that habitat.

Whether F1 hybrids backcrossed to parental species, or crossed with other hybrids, the hypothesis requires a continuous inflow of pollen from *N. fusca* or *N. truncata* from north of the disjunction. Across this distance, this flow must always have been minimal compared to pollen contributed by local *N. solandri* var. *cliffortioides*. This factor distinguishes this situation from that of Potts and Reid (1988), and to a lesser extent from that of Petit et al. (1997), and renders it to some degree less probable. Clearly the hypothesis depends to a large extent on the competitive ability of the very occasional *N. solandri* x *N. truncata* or *N. solandri* x *N. fusca* hybrids greatly exceeding that of the local *N. solandri* individuals in favourable habitats.

8.4 Conclusion

These results demonstrate the value of chloroplast DNA in providing informative markers for population differentiation in *Fuscospora*. This in turn permits inferences on historical distributions, migration patterns and gene flow to be made. However, chloroplast DNA evolution within and among the *Fuscospora* species in New Zealand is very conserved, and does not yield enough variation to allow a full phylogeographic analysis in a study of this size.

The small amount of variation found in this study was however enough to suggest that a barrier to gene flow across the South Island disjunction may have existed for up to 1-2 million years, and that therefore at least one species of *Fuscospora* survived in the far south during the last Pleistocene glacial period. Although this estimate does not allow any convincing resolution between scenarios offered by the 'glacial hypothesis' and 'environmental barriers' hypotheses, it does not provide any support to the 'lateral plate movement' hypothesis. The frequency of haplotype B across the south appears to be uneven, which could support the survival of *Fuscospora* in more than one glacial refugium, each with different frequencies of this haplotype.

Sequence assays from further cpDNA regions in a larger-scale study could detect further polymorphisms which may be phylogenetically informative, and thus allow further inferences on the historical relationship between populations. Additionally, a greater number of polymorphisms differentiating *Fuscospora* populations in the north and south of the South Island will, by reference to *Nothofagus gunnii*, allow a more precise estimate of the age of the disjunction.

Screening for informative RFLP markers would require a much larger project, with PCR amplification of a number of large cpDNA regions (Section 7.4). Identification of a polymorphism detectable by RFLP would be particularly valuable, as it would allow rapid and inexpensive assays of a large number of *Fuscospora* individuals. This would permit more precise determination of the geographical distribution of a haplotype. Population studies using RFLP would also provide information on the structuring of

chloroplast haplotypes at a population level, and therefore on seed-mediated gene flow among populations.

This study has assumed throughout (Section 4.3) the maternal inheritance of cpDNA in *Nothofagus*. The geographic structuring of chloroplast haplotypes found here is consistent with the relatively conserved dispersal mode of seeds, although the results could also be consistent with the more widely scattered dispersal of pollen if the mutation arose recently. By identifying two haplotypes, this study does indicate that the mode of inheritance of chloroplast DNA in *Nothofagus* could be easily determined using genetic techniques. This could be done by assaying progeny of artificial crosses between parents of known, and different, chloroplast haplotypes. Progeny with chloroplast haplotypes identical to their maternal parent would suggest maternal transmission. This approach has already been used to conclude that chloroplast DNA in *Quercus* (Fagaceae) is at least predominantly maternally inherited (Dumolin et al. 1995).

The sharing of chloroplast haplotypes among the three *Fuscospora* species has either been caused by (i) introgression, or (ii) as a consequence of species resurrection via long-distance pollen dispersal and hybridisation. Detection of shared chloroplast haplotypes elsewhere in New Zealand would suggest that chloroplast introgression is prevalent in *Fuscospora*, and that the former hypothesis is feasible. There is an indication that exchange of chloroplast genes may be occuring in the absence of nuclear exchange, which would be consistent with either of these hypotheses. However, existing evidence in relation to nuclear exchange is very limited, and would be enhanced by analysis and comparison of nuclear markers for all three species across a wide geographical range.

A better understanding of the mechanisms bringing about chloroplast introgression in *Fuscospora* can only be gained from empirical study into the hybridisation success of all combinations of the three species. Of great assistance would be data on relative fertility levels and hybrid competitiveness, firstly of F1 hybrids, and secondly of F2 hybrids and backcrosses to each of the parental species. Thus, regardless of whether introgression or species resurrection was involved, this will suggest whether the major role in genetic transfer was played by backcrossing to a particular parent, or by segregation of F2

hybrids. A further line of enquiry is whether unidirectional hybridisation occurs among any combinations of the three species, therefore suggesting whether introgression is directional. Additionally, if chloroplast introgression is occuring in the absence of nuclear introgression, this would help suggest areas of investigation for the causes, for example whether it is associated with synchronisation of flowering times, with floral morphology, or with cytoplasmic male sterility.

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Appendix 1

Details of *Nothofagus* distributions immediately to the north and south of the South Island disjunction.

West of the Main Divide

Nothofagus fusca

North: Common in the upper Grey and northern tributaries of the Ahaura, below 900-1000 metres. Further south, in the southern tributaries of the Ahaura and in the Taramakau, it is found in stands along river terraces and lower slopes (Wardle 1984). Its southern limit is some small stands on the south side of the Taramakau and in some of its southern tributaries: the Otira (to the Deception River) and the Otehake where (along with *N. menziesii*) it is abundant (Wardle 1984)

South: Northern limit is in the Arawata catchment, only occuring in scattered riparian sites in the lower valley, but becoming dominant in the upper valley (Holloway 1954). There are scattered stands and individuals on the lowland hills between the Arawata and Cascade rivers. Red beech is again prevalent further south, in the upper Cascade catchment, and in lowland forest between Gorge River and Big Bay (Wardle 1984).

Nothofagus solandri var. *cliffortioides*

North: Along with *N. menziesii*, *N. solandri* var. *cliffortioides* is the dominant forest tree in the upper tributaries of the Grey (Wardle 1984). It is absent in most of the Ahaura catchment, although there are stands in the Trent and Waiheke tributaries. (Wardle 1970). Its southern limit are stands in the Taramakau and its tributary, the Otehake. There is also a low-altitude stand just south of Lake Brunner (Wardle 1970).

South: Northern limit is in several pockets, near Bald Hill, just north of the Waita River (Holloway 1954), and in the catchments of Grave Creek and Ship Creek (Mark and Lee 1985). Southwards, there is no more record of it until the upper Arawata and

Waiototo rivers (Wardle 1970), and in swamplands between the lower reaches of these two rivers (Wardle 1984). It is quite abundant south-west of the Arawata, between it and the sea (Wardle 1970). Elsewhere, however, in south Westland, its distribution is sporadic, and often limited to infertile and poorly-drained sites (Wardle 1984).

Nothofagus truncata

North: This is abundant in forests to the north of the Ahaura river (Holloway 1954). It is also present on the Paparoa Range, and on the western slopes of the Victoria Range and in the Brunner Range (Wardle 1984). There is no more mention of it in the southern part of the Grey catchment, and its southernmost location is a small isolated stand at Blackwater Creek, just north of the lower Taramakau river, near Kumara (June 1977) - although, if this were the case it would be a tiny outlier, well isolated from the nearest stand of hard beech.

South: Formerly considered to be not present south of the disjunction, June (1977) recorded *N. truncata* at five sites on low granitic hills on the coastal plain between the Waiototo and Arawata rivers. There appear to have been no other reports of *N. truncata* south of the disjunction.

Nothofagus menziesii

North: Along with *N. solandri* var. *cliffortioides*, *N. menziesii* is dominant in the upper tributaries of the Grey and in the northern tributaries of the Ahaura, and is usually the timberline species on the Elliott Range. Its southern limit is the Taramakau. In the Otehake tributary, it dominates the timberline, which is not the case in other tributaries of the Taramakau (Wardle 1984), although there is an isolated stand at 730 m near Otira (Wardle 1967).

South: Its northern limit is the furthest north of all of the beech species, and southward from there it remains the most abundant in south Westland. It is first found on north-facing slopes in the headwaters of the Karangarua catchment, and again in the head of the Mahitahi and Otoko rivers (Wardle 1984). From a 'front' at the Paringa river, southwards it forms extensive pure stands on the slopes of the main ranges, and is occasionally mixed with podocarps in the lowland forest (Holloway

1954). It dominates in the lower reaches of the Arawata valley, and in forests further south.

East of the Main Divide

Nothofagus fusca

North: Common in the headwaters of the Waiau and Hurunui rivers, and its southern limit is in the Waimakariri basin. There are stands in the northern tributaries of the Waimakariri, the Poulter and Hawdon, and on slopes on the northern side of the Waimakariri itself near the confluences with the above tributaries (Holloway 1954). Burrows (1993) records recent establishment (last 60-80 years) of *N. fusca* at Corner Knob, in an area of induced human disturbance on the south side of the Waimakariri, near Cass, although there is no evidence of it in the area in pre-human times. Further east, there are small pockets in the Canterbury foothills, west of Mt Oxford, in the Ashley Gorge, and on Mt Grey (Wardle 1984). There are also several small remnants on the south-east corner of Banks Peninsula.

Over the years there have also been reports of *N. fusca* south of the Waimakariri, although none of these have been confirmed. Holloway (1954) documents unconfirmed reports of a few *N. fusca* in the Moa basin, which drains to the Wilberforce, a tributary of the Rakaia. Wardle (1970) mentions a personal communication made by Holloway of a report of a single *N. fusca* tree in the same area, but it is not clear whether this is the same as Holloway's 1954 report. A specimen of *N. Fusca* (CHR 5827), labelled as collected in the Upper Rangitata in 1869, has been viewed. There have, however, been no other reports of *N. fusca* in the Rangitata catchment.

South: The northernmost confirmed occurrence is a small stand halfway up the Hunter valley, at the northern end of Lake Hawea (Wardle 1984). *N. fusca* is also found at Kidd's Bush, on the northern shores of Lake Hawea, but it is nowhere widespread until further south in the watersheds west of Lake Wanaka (Wardle 1984). Again, there has been a since unconfirmed report of *N. fusca* north of its currently

recognised distribution. Wardle (1984) made mention of a small stand near the head of Lake Ohau, reported by Otago science students in 1958.

Nothofagus solandri var. *cliffortioides*

North: *N. solandri* is the dominant tree species in most of the inland Canterbury ranges and foothills; in fact in most of the region it is the sole large tree species present (Wardle 1970). Towards the southern end of its range, it tends to be absent from the upper reaches of the valleys. Thus it is currently found in tributaries off the lower reaches of the Wilberforce and Mathias rivers, but not further up those rivers (Wardle 1970). It is also in scattered stands in the main Rakaia valley in the vicinity of the Wilberforce and Lake Rivers (Wardle 1970). Further south, stands are small and isolated in the Rangitata catchment. On the northern side of the catchment, it is found between the Lawrence and Potts tributaries, whilst on the southern side it is between the mid-Havelock River and Forest Creek (Wardle 1970). There are isolated stands on and near Mt Peel, and near the headwaters of the Waihi River, a tributary of the Opihi River between Peel Forest and Geraldine (Wardle 1970). The latter appears to be the southernmost stand north of the disjunction.

South: South of the disjunction, *N. solandri* first appears in the Mt Cook area, in tributary creeks of the Tasman river below the Hermitage, particularly nearer Lake Pukaki (Wardle 1970). South of this, it dominates forest in the upper Waitaki and the Clutha tributaries, though not to the main divide where *N. menziesii* dominates (Wardle 1970).

Nothofagus menziesii

Although *N. menziesii* is distinctly disjunct close to the divide, the presence of *N. menziesii* in an irregular pattern of patches right down the east side of the island brings into question whether a single disjunction characterises the distribution of this species. Although the patches are well isolated from one another, nowhere is there a single gap which could be readily defined as the dividing line for a north-south disjunction. Burrows (1965) recognised this, and classified *N. menziesii* among his 'partially disjunct' species.

North: *N. menziesii* is rather less abundant to the east of the divide than it is to the west. It is common in the head of the Waiau and Hurunui rivers, but south of that its distribution tends to be very patchy and characterised by small isolated stands. In the Waimakariri catchment, it is restricted to the upper Poulter (Wardle 1970) and Hawdon (Wardle 1967) tributaries. In the Rakaia, it only occurs in the Lake Stream tributary, with no more records within 40 kilometres (Wardle 1984). Southwards again, there are a few individuals on the eastern slopes of Mt Somers, and to the west of the mountain there are stands in the Woolshed Creek tributary of the South Ashburton River, where it co-dominates with *N. solandri* var. *cliffortioides*. Holloway (1954) reported a single veteran specimen further inland, in a *N. solandri* var. *cliffortioides* pocket on the Arrowsmith Range. The southernmost record is of a small stand, again very isolated from all others, near Burke Pass (Wardle 1967).

South: In a similar pattern to that immediately north of the disjunction, the distribution of *N. menziesii* to the south is very patchy. Along with *N. solandri* var. *cliffortioides*, it reappears in the Mt Cook area, in tributaries of the Tasman River (Wardle 1970). It is the sole beech species in Governors Bush near the Hermitage (Holloway 1954). South of there, in the headwaters of the Waitaki catchment, it is limited only to the very top reaches of the Dobson and Hopkins valleys, near the divide, but is more abundant at the head of the Hunter (Wardle 1970). It is the only beech species found further to the east. There are stands in the Hunter Hills, near Cave; in the Waianakarua catchment between Oamaru and Palmerston; and further south in eastern Otago (Wardle 1984).

Nothofagus truncata

This species does not occur east of the divide, south of the Wairau River in Marlborough (Wardle 1984).

Appendix 2

cpDNA regions sequenced in this project

1. *trn*L intron and *trn*L-*trn*F intergenic spacer

Haplotype A

```
0    GACTTAATTG GGTTGAGCCT TGGTATGGAA ACCTACCAAG TGATAACTTT
50   CAAATTCAGA GAAACCCCGG AATTAAAAAT GGGCAATCCT GAGCCAAATC
100  CTGTTTTTCG AAAACAAATT AAATAAAGAT TCAGTTCAGA AAGCGAGAAT
150  AAAAAAAGGA TAGGTGCAGA GACTCAATGG AAGCTGTTCT AACAAATGGA
200  GTTGACTACC TTGCGTTAGT AAAAGAATCC TTCTATCAAA ACTACAGTAA
250  GGGGTGAAGT ATAAACCTAT ATACATAGGT ATACGTACTG AAATACTATC
300  TCAAATGATT AATGACGACC CAAATCGAAT CTTTATTAAT CAAATCATTT
350  ACTCCATCAT AGTCTGATAT ATCTTTTGAA GAACTAATGA ATCGGACGAG
400  AATAAAGATA GAGTCCCATT CTACATGTTA ATACCGACAA CAATGAAACT
450  TAGAGTAAGA GGAAAATCCG TCGACTTTAG AAATCGTGAG GGTTCAAGTC
500  CCTCTATCCC CAAAAAGGCC CGTTTGACTC CCTAATTATT TATCCGATCC
550  GCCCTTTTCG TTAATGGTTC AAAATTCATT ATCTTTCTCA TTCATTCTAC
600  TCTTTTACAA AATACAATAA AGAAAGGGTC TGAGTGGAAA TTTTTTTTCT
650  TATCACAATA CTTGTAATAT ATATGATACA CGGACAAACG AGCATTTTTA
700  TCTTTGAGCA AGGTATCCCC ATATTCCAAT TTTAATGATT AACAATACGT
750  ATCATTCCTC GTACTGTACT GAAACTTACA AAGTTTTCTT TTTGAAGATC
800  CAAGAAATTC CAGGGCCTGA ATAAGACTTT GTAATACTTT TTTTCTTTTT
850  AATTGACATA AACCCAAGTC ATCTATTAAA ATAAGGATGG TGCGCCGGGA
900  GTGGTCGGGA TAGCTCAGCT GGTAGAGCAG AGGACTGAAA ATC
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Haplotype B

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0    GACTTAATTG GGTTGAGCCT TGGTATGGAA ACCTACCAAG TGATAACTTT
50   CAAATTCAGA GAAACCCCGG AATTAAAAAT GGGCAATCCT GAGCCAAATC
100  CTGTTTTTCG AAAACAAATT AAATAAAGAT TCAGTTCAGA AAGCGAGAAT
150  AAAAAAAGGA TAGGTGCAGA GACTCAATGG AAGCTGTTCT AACAAATGGA
200  GTTGACTACC TTGCGTTAGT AAAAGAATCC TTCTATCAAA ACTACAGTAA
250  GGGGTGAAGT ATAAACCTAT ATACATAGGT ATACGTACTG AAATACTATC
300  TCAAATGATT AATGACGACC CAAATCGAAT CTTTATTAAT CAAATCATTT
350  ACTCCATCAT AGTCTGATAT ATCTTTTGAA GAACTAATGA ATCGGACGAG
400  AATAAAGATA GAGTCCCATT CTACATGTTA ATACCGACAA CAATGAAACT
450  TAGAGTAAGA GGAAAATCCG TCGACTTTAG AAATCGTGAG GGTTCAAGTC
500  CCTCTATCCC CAAAAAGGCC CGTTTGACTC CCTAATTATT TATCCGATCC
550  GCCCTTTTCG TTAATGGTTC AAAATTCATT ATCTTTCTCA TTCATTCTAC
600  TCTTTTACAA AATACAATAA AGAAAGGGTC TGAGTGGAAA TTTTTTTTCT
650  TATCACAATA CTTGTAATAT ATATGATACA CGGACAAACG AGCATTTTTA
700  TCTTTGAGCA AGGTATCCCC ATATTCCAAT TTTAATGATT AACAATACGT
750  ATCATTCCTC GTACTGTACT GAAACTTACA AAGTTTTCTT TTTGAAGATC
800  CAAGAAATTC CAGGGCCTGA ATAAGACTTT GTAATACTTT TTTTCTTTTT
850  TAATTGACAT AAACCCAAGT CATCTATTAA AATAAGGATG GTGCGCCGGG
900  AGTGGTCGGG ATAGCTCAGC TGGTAGAGCA GAGGACTGAA AATC
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2. *trn*E-*trn*T intergenic region

```
0     ATACTTGCCC GACCGCCACC ATACTATGCT CATAATATGA ACAGTTTTTT
50    AAAATTGTCA ATATAATGGT ATGACTCAAT TTGAAGGATC TTTCCCTCTT
100   TCATGATTCT ATAGAATCTT TTTTTTCTCT AATAATTTAG TCCAGGGGGA
150   CAAATAAAAT CTTTTTATCA ACGATTCGAT TAAATATCAT AGATCTATGT
200   TTAATTGGTA AGCATATGTA TCAATTAAAT AAATTTCGTT ATGATGGGGG
250   TCAATTCAAT CAATTAGGGT TGGCCTTGAA ACAATTCATT CCATTGCTAT
300   TTATTAAATC CAATCTTACT AAACCATTTT TTATTTTACC TATACTTACT
350   AGATAAATTA TATTTCTCGT TCCTGAATGG TCTACTATGC GGATAATCAC
400   TATGCAGATA AGATATACCT ATGTACAATA AAATATATCT ATGTACATAT
450   ATTATAATAA AATAAGTATA TGCACTGGCT TCATAGTGGC TAATGGCTTA
500   TTCAGTAATT GAATCAAATG GG
```

3. *trn*F-*ndh*J intergenic region

```
0     CTGGTTCTGG CACATGATCA ATTTGTATGC ATAGCCATTC TACAAATTAA
50    TTGATATGAG TCGACATATA TATTCATTAC AGTAAGAGTA TATCTCATGA
100   TACATACTTA TCTATCTGGC CGTTTGAGGA TATGTCTGCC CTTTATAGAA
150   TATAAATAAA ATAGAATAAA TGAGTAAAGG GTATATGTAT ATAAAGTAAT
200   AAGCTATGTA AAATAAAAGA ATTCGATTTT TTCCTCTTCT TTTTTATTTG
250   TTCATATTAT ATTGTGTCTT TTCTTGTTCA AAAACGAATG TTAAGACTTC
300   ATACATATTA GAATAAATTA AATTAGTTGG TTGAAAGACT CAAAAGTCTA
350   GTCTAGAGGA GTTGAAGGGT GGGAATAGTC AAGATTCATC TCAGATACAG
400   TACAAATAGA ATCCGATCCC CTTTTCATTT CTTTATATTT TTTTTCCTTT
450   CATATTCTAT TTCTTCACTT CCTCTTATGT AACTTTCTAG AGCCCATCTA
500   AATGATGGGC GCGGTACATA GTTCATGATG CAGAACTCTT TTAGTTCATC
550   CTATTGGCAA TGAGGCTTCA AAAACTCTGT TTGATCTATA AGAGAACATA
600   CAAATATTAT TTGAGTATCA GGAGTTGTAG AAGTAAAGAT TAG
```

*4 trn*K-*mat*K intergenic region

```
0     TAGAGTACTC GGCTTTTAAG TGCGGCTAGC ATCTTTTACA CATTTGTATG
50    AAGCAAGGGA TTCGTCGATA CCATCGGTAG AGCTTGTAAG ACCACGACTG
100   ATCCTGAAAG GAATGAATGG AAAAAGCAGC ATGTCGTATC AATGGAGAAT
150   TATAAGAATA TTTGATTCTT ACTGGATCGG TCAAAAACCT TGTGTTTGAA
200   TTTTTGACGC GAAAAAAAGA AAATAATAAA TTTTAAAATT TTAAGAAAAT
250   TGGGTCAAGT GAATAAATGG ATAGAGCCCT ATGGCTCCAA TTAGAGGGAA
300   ATAAAAAGCA ACGGGCTTCT GTTCTTAATT TGAATGATTA CCCGATCTAA
350   TTCGACGTTA AAAATATATT AGCGCTTGAT GCGGGAAATG TTTTTCCCAT
400   GAGTAGATTA TCGATTTTTT TTATGAATCC TAATTCTATT CTCCATTATG
450   GTGTGGGGAT GAATGTGTAG AAGAAGCAGT ATATTGATAA CAAGATTTTT
500   GTTTCCAAAA TCAAAAGATC GATTGGGTTG AAAAAATAAA GGATTTCTGA
550   CCATCTTGTT ATCCCTAAAA TGAACATAAA CCAATTAAAT TAGATGTAAA
600   AAGAGAGGAT AGAGAGTCCG TTGATGAGTC TTATCTGTTT CTGGGGTATC
650   TATTCTTTTC TTACTATAAT ACCTTGTTTT GACTGTATCG TACTATGTGT
700   CATTTAATAA CCCAAATTTT ATAACCCAAT AAATCCCCTA TCCCGGTCTC
750   AAATCTAATT TCAAAATGGA GGAATTTCAA GGATATTTAG AATTAGATAA
800   ATTTAGGCAA CATGACTTCC TATACCCACT TATCCTTCGG GAGTATATTT
850   ATGCACTTGC TCACGATCAT GGTTTAAATA GATCGATTTT ATTAGAAAAT
900   TTTGGTTATG ATAATAAATA TAGTTTACTA AATGTAAAAC GTTTAATTGC
950   T
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